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(54) Title: MODIFIED BACILLUS ANTHRACIS VACCINE COMPOSITIONS AND METHODS OF USE THEREOF

(57) Abstract: A variety of modified *Bacillus anthracis* bacteria useful in vaccines are provided. For instance, asporogenic strains of*Bacillus anthracis* are provided. In addition, *Bacillus anthracis* strains attenuated in their ability to repair their nucleic acid, such as in their nucleic acid excision repair ability or recombination repair ability, are provided. Strains expressing an antigen, such as protective antigen, under the control of a heterologous promoter and/or an inducible promoter are also provided. *Bacillus anthracis* bacteria comprising mutations in toxin genes are further provided. Vaccine compositions comprising the bacteria, methods of making the modified strains, and methods of using the vaccines are also provided.

MODIFIED BACILLUS ANTHRACIS, VACCINE COMPOSITIONS AND METHODS OF USE THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the priority benefit of U.S. Provisional Application No. 60/599,522, filed August 5, 2004, and U.S. Provisional Application Serial No. 60/584,886, filed June 30, 2004, the disclosures of each of which are hereby incorporated by reference herein in their entirety. This application is also a continuation-in-part of the prior application U.S. Patent Application Serial No. 10/883,599, filed June 30, 2004, which claims priority to U.S. Patent Application Serial No. 10/773,618, filed February 6, 2004, the disclosures of each of which are hereby incorporated by reference herein in their entirety. This application is also a continuation-in-part of the prior application International Application No. PCT/US2004/23881, filed July 23, 2004, the disclosures of which are hereby incorporated by reference herein in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

[0002] This invention was made, in part, with government support under grant number 1U01AIO61199-01, awarded by the National Institutes of Health. The government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] The present invention relates generally to vaccine compositions and immunotherapy. In particular, the present invention relates to vaccine compositions comprising modified *Bacillus anthracis* bacteria.

BACKGROUND OF THE INVENTION

[0004] A variety of vaccines have been developed for clinical use, mostly targeting the prevention of infectious diseases caused by viruses, bacteria and parasites. Vaccines can be prepared from live attenuated microbes, inactivated (killed) microbes, or components of the microbes themselves. Live attenuated microbes contain genetic alterations, such as deletion or modification of virulence factors, resulting in a less virulent microbe. For inactivated vaccines, a microbe may be chemically or physically inactivated. Ideally, such vaccines cannot cause an infection but are still able to stimulate a desired immune response. Examples of inactivated vaccines include polio and influenza viruses, and bacterial vaccines against cholera and pertussis, although live attenuated vaccines are an option for polio, influenza, and cholera as well. In order to elicit the desired immune response, it is important that the inactivated microbe comprises the appropriate antigens prior to inactivation. It has been observed in some cases that inactivating the microbe results in a significantly reduced immune response because *de novo* gene expression by an infecting microbe is required to stimulate an optimal immune response. Methods that have been used to inactivate bacteria include the use of acetone, alcohol, formalin, glutaraldehyde, paraformaldehyde, or phenol, heating, or ultraviolet irradiation (Pace et al., *Vaccine* 16:1563-1574 (1998)).

[0005] Efforts to develop a safe, effective vaccine against one deadly agent, *Bacillus anthracis*, using traditional technologies have been largely unsuccessful. The dormant and extremely durable spore form of *Bacillus anthracis*, the causative agent of anthrax, is an ideal biological weapon (World Health Organization. Health aspects of chemical and biological weapons: a report of a WHO group of consultants. Geneva Switzerland: World Health Organization; Mock, M. and A. Fouet, *Annu. Rev. Microbiol.* 55:647-671(2001)). Inhaled spores are transported by alveolar macrophages to the lymph nodes surrounding the lungs, where they germinate, multiply, and produce high levels of cytotoxins, killing up to 99% of immunologically naïve victims who don't receive antibitiotic therapy. The only licensed human anthrax vaccine, anthrax vaccine absorbed (AVA), was developed in the late 1950s and is poorly immunogenic (Report, Case Report: Use of Anthrax Vaccine in the United States: Recommendations of the Advisory Committee on Immunization Practices, in Clinical Toxicology. 2001. p. 85-100). The prolonged 18-month vaccination regimen and required

annual boosters are problematic for immunization of military personnel both in terms of safety and in terms of practicality. AVA is based on processed *B. anthracis* culture supernatants containing various amounts of lethal factor (LF) (encoded by the *lef* gene), edema factor (EF) (encoded by the *cya* gene), and protective antigen (PA) (encoded by the *pagA* gene) formulated with an adjuvant. In addition to being weakly immunogenic, AVA represents only a small fraction of the expressed bacterial proteins and does not stimulate immunity against other known *B. anthracis* virulence determinants, for example capsule. Thus, the possibility of new strains strategically engineered to subvert the present vaccine constitutes a genuine threat. Given the development and use around the world of *B. anthracis* spores as a biological weapon, there is a clear need for a safe, effective anthrax vaccine that combines safety with potency to elicit broad and durable protective immune responses in vaccinated individuals.

SUMMARY OF THE INVENTION

[0006] The invention provides a variety of modified *Bacillus anthracis* bacteria suitable for use in vaccines. In some embodiments, the modified *Bacillus anthracis* bacteria comprise mutations that attenuate the ability of the bacteria to repair its nucleic acid. In some embodiments, the modified *Bacillus anthracis* are asporogenic or sporulation-deficient. Modified *Bacillus anthracis* bacteria expressing an antigen, such as protective antigen, under the control of a heterologous promoter and/or an inducible promoter are also provided. Modified *Bacillus anthracis* bacteria comprising mutations in toxin genes are further provided. In some embodiments, the modified bacteria have decreased toxicity, and/or have increased immunogenicity. In some embodiments, the ability of the modified *Bacillus anthracis* to proliferate is attenuated (preferably while sufficient gene expression is maintained). The invention also provides methods for modifying *Bacillus anthracis* for use in vaccines. The invention further provides immunogenic compositions and vaccine compositions comprising the modified *Bacillus anthracis*. Methods of using the modified bacteria and compositions comprising the bacteria for the induction of immune responses in a host and/or for the prevention of disease are also provided.

[0007] In one aspect, the present invention provides modified *Bacillus anthracis* bacteria, and compositions thereof (e.g., vaccine compositions), which are capable of generating an

immune response upon administration to a host. In some embodiments, the immune response is a response which protects the host from a disease related to infection by *Bacillus anthracis*, such as anthrax. In some embodiments, the immune response comprises a CD4+ immune response, a CD8+ immune response, or both a CD4+ and CD8+ immune response. In some embodiments, the immune response comprises an immune response specific to lethal factor (LF), edema factor (EF), protective antigen (PA), capsule, and/or whole bacteria. In some embodiments, the bacteria are defective with respect to at least one nucleic acid repair enzyme. In some embodiments, the nucleic acid of the bacteria has been modified so that the bacteria are attenuated for proliferation. Methods of using the bacteria and compositions to generate an immune response in a host to *Bacillus anthracis* and/or protect a host from disease comprising administering an effective amount of the bacteria and/or compositions to the host are further provided.

[0008] In another aspect, the present invention provides a *Bacillus anthracis* strain that is attenuated for nucleic acid repair. In some embodiments, the strain is defective with respect to at least one DNA repair enzyme. In some embodiments, the DNA repair enzyme is UvrA, UvrB, UvrC and/or RecA. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuate for proliferation. In some embodiments, the nucleic acid of the bacteria has been modified by reaction with a nucleic acid targeting compound that reacts directly with nucleic acid, so that the bacteria are attenuated for proliferation. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0009] In another aspect, the present invention provides a *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation. In some embodiments, the nucleic acid of the bacterium has been modified by reaction with a nucleic acid targeting compound that reacts directly with nucleic acid, so that the bacterium is attenuated for proliferation. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacterium are provided. Methods of inducing an immune

response in a host comprising administering an effective amount of a composition comprising the modified bacterium are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising the modified bacteria.

[0010] In another aspect, the present invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleotide excision repair (NER). In some embodiments, the strain comprises an inhibiting mutation, or a modification that attenuates expression of, at least one sporulation gene. In some embodiments, the strain is defective with respect to SpoIIE. In some embodiments, the strain comprises a mutation in the *spoIIE* gene. Moreover, in some embodiments, the strain is defective with respect to one or more enzymes selected from the group consisting of UvrA, UvrB, and UvrC. In some embodiments, the strain is defective with respect to SpoIIE, UvrA, and UvrB. In some embodiments, the strain comprises a mutation in at least one DNA nucleotide excision repair gene. For instance, in some embodiments, the strain comprises a mutation in one or more genes selected from the group consisting of *uvrA* gene, *uvrB* gene, and *uvrC* gene. In some embodiments, the strain further comprises a mutation in, or a modification that attenuates expression of, a recombinational repair gene, such as *recA*. In some embodiments, the mutation or modification comprises a conditional mutation or a transcriptional or translational activator or repressor. In some embodiments, the mutation comprises a conditional mutation. In some embodiments, the modification comprises a nucleic acid molecule encoding a RecA protein, wherein expression of the RecA protein is under the control of a transcriptional or translational activator or repressor (e.g., a transcriptional repressor such as the lac repressor). In some embodiments, the strain is defective with respect to RecA (e.g., comprises a mutant *recA* gene which encodes a temperature-sensitive RecA or an inducible or repressible *recA* mutant). In some embodiments, the strain further comprises an inhibiting mutation, or a modification that attenuates expression of, at least one toxin gene. In some embodiments, the at least one toxin gene comprises the *lef* gene, *cya* gene, or *lef* and *cya* genes. In some embodiments, the strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes, that decreases the toxicity of the strain. In some embodiments, the strain further comprises a nucleic acid encoding at least one antigen, wherein the nucleic acid encoding the at least one antigen is operably linked to a heterologous promoter. In some embodiments, the heterologous promoter is inducible. For instance, the promoter is, in some embodiments,

inducible by ultraviolet light, a nucleic acid targeted compound, a nucleic acid cross-linking compound, and/or an SOS regulatory pathway. In some embodiments, the antigen is protective antigen and the nucleic acid encoding the protective antigen is operably linked to an SOS regulatory sequence. In some embodiments, the nucleic acid encoding the at least one antigen is, or is derived from, a gene selected from the group consisting of the following: *pagA* gene; *lef* gene; *cya* gene; *pagA* and *lef* genes; *pagA* and *cya* genes; *cya* and *lef* genes; and *pagA*, *lef*, and *cya* genes. In some embodiments, the strain comprises a poly-D-glutamate capsule, while in some alternative embodiments the strain lacks a poly-D-glutamate capsule. in some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. By way of non-limiting example, the nucleic acid may have been modified by reaction with a nucleic acid targeting compound that reacts directly with the nucleic acid (e.g., the nucleic acid has been treated with a psoralen and UVA radiation). A bacterium of the strain is also provided which further comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. A vaccine or composition comprising the *Bacillus anthracis* strain is also provided, as well as a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of the strain.

[0011] In yet another aspect, the invention provides a sporulation-deficient *Bacillus anthracis* strain that is attenuated for nucleotide excision repair (NER). In some embodiments, the strain comprises an inhibiting mutation, or a modification that attenuates expression of, at least one sporulation gene. In some embodiments, the strain is defective with respect to SpoIIE. In some embodiments, the strain comprises a mutation in the *spoIIE* gene. Moreover, in some embodiments, the strain is defective with respect to one or more enzymes selected from the group consisting of UvrA, UvrB, and UvrC. In some embodiments, the strain comprises a mutation in at least one DNA nucleotide excision repair gene. For instance, in some embodiments, the strain comprises a mutation in one or more genes selected from the group consisting of *uvrA* gene, *uvrB* gene, and *uvrC* gene. In some embodiments, the strain further comprises a mutation in, or a modification that attenuates expression of, a recombinational repair gene, such as *recA*. In some embodiments, the mutation or modification comprises a conditional mutation or a transcriptional or translational activator or repressor. In some embodiments, the

mutation comprises a conditional mutation. In some embodiments, the modification comprises a nucleic acid molecule that encodes a RecA protein, wherein expression of the RecA protein is under the control of a transcriptional or translational activator or repressor (e.g., the lac repressor). In some embodiments, the strain further comprises an inhibiting mutation, or a modification that attenuates expression of, at least one toxin gene. In some embodiments, the at least one toxin gene comprises the *lef* gene, *cya* gene, or *lef* and *cya* genes. In some embodiments, the strain comprises a nucleic acid encoding at least one antigen, wherein the nucleic acid encoding the at least one antigen is operably linked to a heterologous promoter. In some embodiments, the heterologous promoter is inducible. For instance, the promoter is, in some embodiments, inducible by ultraviolet light, a nucleic acid targeted compound, a nucleic acid cross-linking compound, and/or an SOS regulatory pathway. In some embodiments, the nucleic acid encoding the at least one antigen is or is derived from a gene selected from the group consisting of the following: *pagA* gene; *lef* gene; *cya* gene; *pagA* and *lef* genes; *pagA* and *cya* genes; *cya* and *lef* genes; and *pagA*, *lef*, and *cya* genes. In some embodiments, the strain comprises a poly-D-glutamate capsule, while in some alternative embodiments the strain lacks a poly-D-glutamate capsule. A bacterium of the strain is also provided which further comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. A vaccine or composition comprising the *Bacillus anthracis* strain is also provided, as well as a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of the strain.

[0012] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a nucleic acid encoding an antigen, wherein the nucleic acid encoding the antigen is operably linked to a heterologous promoter. In some embodiments, the nucleic acid encoding the antigen is, or is derived from a gene selected from, the group consisting of the following: *pagA* gene; *lef* gene; *cya* gene; *pagA* and *lef* genes; *pagA* and *cya* genes; *cya* and *lef* genes; and *pagA*, *lef*, and *cya* genes. In some embodiments, the heterologous promoter is inducible. In some embodiments, the heterologous promoter is inducible by ultraviolet light, a nucleic acid cross-linking compound, ultraviolet light and a nucleic acid cross-linking compound, an SOS regulatory pathway, and/or a change or shift in temperature. The invention further provides a vaccine or composition comprising a bacterium of the *Bacillus anthracis* strain. The present

invention also provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to a host an effective amount of a composition comprising a bacterium of the strain.

[0013] In an additional aspect, the invention provides a *Bacillus anthracis* strain comprising at least one inhibiting mutation in each of *uvrA* gene, *uvrB* gene, *lef* gene, *cya* gene, and *spoIIE* gene. Also provided is a bacterium of the *Bacillus anthracis* strain, wherein the bacterium further comprises at least one covalently linked nucleic acid cross-linking compound linked to the genomic DNA of the bacterium. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0014] In still another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair. In one embodiment, the strain is defective with respect to SpoIIE. In some embodiments the strain is also defective with respect to UvrA and/or UvrB (e.g., comprises a mutation in the *uvrAB* genes). In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments the strain is also defective with respect to RecA (e.g., wholly defective, partially defective, or conditionally defective with respect to RecA). In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. In some embodiments, the *Bacillus anthracis* strain is a pXO1+ and pXO2+ strain. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from disease (i.e., a disease caused by infection with *B. anthracis*), comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0015] In another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair, wherein the strain is defective with respect to at least one DNA repair enzyme selected from the group consisting of UvrA, UvrB, UvrC, and RecA. In some embodiments, the bacterium of the *Bacillus anthracis* strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the nucleic acid has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid (e.g., a psoralen compound activated by UVA irradiation), so that the bacterium is attenuated for proliferation. In some embodiments, the bacterium is defective with respect to SpoIIE. For example, in some embodiments the bacterium comprises a mutation in the *spoIIE* gene which renders the bacterium defective with respect to SpoIIE. In some embodiments, the strain is defective with respect to UvrA, UvrB, or both UvrA and UvrB. For instance, in some embodiments, the strain comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene. In some embodiments, the strain is defective with respect to SpoIIE, UvrA, and UvrB. In some embodiments, the strain is defective with respect to UvrC (e.g., the strain comprises a mutation in the *uvrC* gene). In some embodiments, the strain is defective with respect to RecA. In some embodiments, the strain comprises a mutation in the *recA* gene. In other embodiments, the strain is a repressible *recA* mutant. In some embodiments, the strain comprises a *recA* gene that is under the control of the lac repressor. In further embodiments, the strain comprises a temperature sensitive *recA* gene. In some additional embodiments, the strain expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0016] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a heterologous expression cassette comprising a sequence encoding protective antigen (PA), wherein the sequence encoding protective antigen is operably linked to an inducible promoter. In some embodiments, the expression of the protective antigen is induced by treatment of the

strain with a psoralen and UVA radiation. For instance, in some embodiments, the nucleic acid encoding protective antigen is operably linked to an SOS regulatory sequence, or any promoter that is induced in response to photochemical treatment or other modification of the DNA of the bacterium. In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, at least one nucleic acid repair gene (e.g., a nucleotide excision repair (NER) gene, a recombinational repair gene, or an NER gene and a recombinational repair gene). In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, a nucleic acid repair gene that is selected from the group consisting of *uvrA*; *uvrB*; *uvrC*; *uvrA* and *uvrB*; and *uvrA*, *uvrB*, and *uvrC*. In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, *recA*. In some embodiments, the mutation or modification comprises a conditional mutation or a transcriptional or translational activator or repressor. In some embodiments, the mutation comprises a conditional mutation. In some embodiments, the modification comprises a nucleic acid molecule that encodes a RecA protein, wherein expression of the RecA protein is under the control of a transcriptional or translational activator or repressor (e.g., a transcriptional repressor, such as the lac repressor). In some embodiments, the strain comprises a mutation that attenuates the ability of the strain to repair its nucleic acid. For instance, in some embodiments, the strain is defective with respect to at least one DNA repair enzyme, such as UvrA, UvrB, or both UvrA and UvrB. In some embodiments, the strain comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene. In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments, the strain is defective with respect to RecA. A strain that is defective with respect to RecA can, by way of example, comprise a mutation in its *recA* gene, comprise a *recA* gene under control of a repressible promoter, or comprise a temperature sensitive *recA* gene. In some embodiments, the strain is a repressible *recA* mutant. In some embodiments, the strain comprises a *recA* gene that is under the control of the lac repressor. In some embodiments, the strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the nucleic acid of the bacteria has been modified by reaction with a nucleic acid targeting compound that reacts directly with the nucleic acid (e.g., a psoralen compound

activated by UVA irradiation). In some embodiments, the bacteria of the strain further comprise at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacteria. In addition, the strain is, in some embodiments, asporogenic (e.g., is defective with respect to SpoIIE, such as a *spoIIE* mutant). In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, at least one sporulation gene or toxin gene (e.g., *spoIIE* gene; *lef* gene; *cya* gene; *spoIIE* and *lef* genes; *spoIIE* and *cya* genes; *lef* and *cya* genes; *spoIIE*, *lef*, and *cya* genes). In some embodiments, the strain comprises a poly-D-glutamate capsule, whereas in other embodiments, the strain lacks a poly-D-glutamate capsule. Vaccines and compositions comprising bacteria of the strain are also provided. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of the strain is also provided. A method of protecting a host from a disease, comprising administering to the host an effective amount of a composition (e.g., a vaccine) comprising a bacterium from the strain is also provided.

[0017] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the *Bacillus anthracis* strain is attenuated for nucleic acid repair (e.g., is defective for UvrA, UvrB, UvrC, and/or RecA). In some embodiments, the strain is asporogenic. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0018] In yet another aspect, the invention provides a *Bacillus anthracis* strain that is defective with respect to *recA*. In some embodiments, the strain is a conditional *recA* mutant. In some embodiments, the strain is a temperature sensitive *recA* mutant. In some embodiments, the strain is a repressible *recA* mutant. In some embodiments, the strain comprises an expression

cassette which expresses RecA antisense RNA. In some embodiments, the strain is an inducible *recA* mutant. In some embodiments, the *recA* gene is under control of a lac repressor. In some embodiments, the strain comprises a *recA* gene that is operably linked to a repressible promoter. In some embodiments, the strain comprises a heterologous expression cassette encoding protective antigen, wherein the protective antigen is operably linked to an SOS regulatory sequence. In some embodiments, the strain is defective with respect to at least one additional DNA repair enzyme such as UvrA, UvrB, or both UvrA and UvrB. (For instance, the strain optionally comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene.) In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments, the modified strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. In some embodiments, the nucleic acid of the bacteria of the strain has been modified (e.g., by a nucleic acid targeting compound that reacts directly with the nucleic acid, such as a psoralen compound activated by UVA irradiation), so that the bacteria are attenuated for proliferation. In some embodiments, the *Bacillus anthracis* strain is asporogenic (e.g., defective with respect to SpoIIE, such as *spoIIE* mutant). Compositions, such as vaccines, comprising a bacterium from the *Bacillus anthracis* strain are also provided. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of the strain is also provided. A method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain is further provided.

[0019] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a temperature-sensitive *recA* gene. In some embodiments, the strain comprises a *recA* gene which comprises a mutation analogous to the V246M mutation of the recA44 temperature-sensitive *recA* mutant of *E. coli*. For instance, in some embodiments, the bacteria of the strain comprise a mutation in a *Bacillus anthracis recA* gene, wherein the *recA* gene encodes a temperature-sensitive RecA protein comprising a V244M mutation. In some embodiments, the bacteria of the strain comprise a *recA* gene derived from a foreign bacterium, such as *E. coli*, wherein the *recA* gene encodes a temperature-sensitive RecA protein. In some embodiments, the bacteria comprise a *recA* gene derived from *E. coli*, wherein the *recA* gene comprises a mutation analogous to the V246M mutation of the *E. coli* recA44 temperature-sensitive *recA* mutant. In some

embodiments, the strain comprises a *recA* gene that comprises the recA44(ts) allele of *E. Coli*. In some embodiments, the strain is defective with respect to UvrA and/or UvrB (e.g., comprises a mutation in the *uvrA* and/or *uvrB* gene). In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments, the strain is asporogenic. In some embodiments, the strain comprises a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0020] In an additional aspect, the invention provides a *Bacillus anthracis* bacterium which is a repressible or inducible *recA* mutant. In some embodiments, the bacterium is a repressible *recA* mutant. In other embodiments, the bacterium is an inducible *recA* mutant. In some embodiments, the bacterium comprises a nucleic acid encoding a RecA protein, wherein expression of the RecA protein is under the control of a heterologous transcriptional repressor or activator. In some embodiments, the bacterium comprises a *recA* gene, wherein expression of the *recA* gene is under control of a lac repressor. In some embodiments, the bacterium comprises a nucleic acid encoding a RecA protein, wherein the nucleic acid is operably linked to a heterologous operator which binds a repressor or activator. In some embodiments, the bacterium comprises a nucleic acid that produces a RecA antisense RNA upon transcription, wherein the nucleic acid is operably linked to an operator which binds a repressor or activator. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising the bacteria are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising the bacteria. Methods of protecting a host from a disease

comprising administering an effective amount of a composition comprising the bacteria are also provided.

[0021] In another aspect, the invention provides a *Bacillus anthracis* strain attenuated for nucleic acid repair, wherein the strain is defective with respect to RecA and at least one additional DNA repair enzyme selected from the group consisting of UvrA, UvrB, and UvrC. Compositions, such as vaccine compositions, comprising the bacterium of the strain are also provided. Methods of using compositions comprising the strain are also provided.

[0022] In another aspect, the invention provides an asporogenic *Bacillus anthracis* strain attenuated for nucleic acid repair, wherein the strain is defective with respect to SpoIIE and at least one additional DNA repair enzyme selected from the group consisting of UvrA, UvrB, and UvrC. Compositions, such as vaccine compositions, comprising the bacterium of the strain are also provided. Methods of using compositions comprising the strain are also provided.

[0023] In still another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair, comprising a temperature sensitive *recA* gene and a mutation in *uvrA*, *uvrB*, or *uvrC* (e.g., a deletion of *uvrA*, *uvrB*, or *uvrC*). Compositions, such as vaccine compositions, comprising the bacterium of the strain are also provided. Methods of using compositions comprising the strain are also provided.

[0024] In yet another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair, comprising a repressible *recA* gene and a mutation in *uvrA*, *uvrB*, or u*vrC* (e.g., a deletion of *uvrA*, *uvrB*, or *uvrC*). Compositions, such as vaccine compositions, comprising the bacterium of the strain are also provided. Methods of using compositions comprising the strain are also provided.

[0025] In another aspect, the invention provides an asporogenic *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation. In some embodiments, the bacterium has been modified by reaction with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation. In some embodiments, the nucleic-acid targeted compound is a nucleic acid alkylator. In some embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., a psoralen compound activated by UVA irradiation). In some embodiments, the bacterium comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. In some

embodiments, the bacterium is defective with respect to SpoIIE. In some embodiments, the bacterium comprises a mutation in a sporulation gene, such as, but not limited to, *spoIIE*. In some embodiments, the bacterium is attenuated for nucleic acid repair. For instance, in some embodiments, the bacterium is defective with respect to at least one DNA repair enzyme (e.g., UvrA, UvrB, and/or UvrC). In some embodiments, the bacterium comprises a mutation in one or more genes selected from the group consisting of *uvrA*, *uvrB*, and *uvrC*. In some embodiments, the bacterium is defective with respect to SpoIIE, UvrA, and UvrB. In some embodiments, the bacterium is attenuated for recombinational repair. For instance, in some embodiments, the bacterium is defective with respect to RecA. In some embodiments, the bacterium comprises a mutation in the *recA* gene. In some embodiments, the bacterium comprises a *recA* gene that is under the control of the lac repressor. For instance, in some embodiments, the bacterium comprises a mutant *recA* gene which encodes a temperature-sensitive RecA. In some embodiments, the bacterium is a repressible or inducible *recA* mutant. In some embodiments, the bacterium expresses protective antigen (PA) under the control of an SOS regulatory sequence. In some embodiments, the bacterium comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacterium are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising the bacterium are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising the bacterium.

[0026] In another aspect, the invention provides an asporogenic *Bacillus anthracis* bacterium that is attenuated for nucleic acid repair, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation. In some embodiments, the bacterium has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation. In some embodiments, the nucleic-acid targeted compound is a nucleic acid alkylator. In some embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., a psoralen compound activated by UVA irradiation). In some embodiments, the bacterium is defective with respect to SpoIIE. In some embodiments, the bacterium comprises a mutation in a sporulation gene, such as, but not limited to, *spoIIE*. In some embodiments, the bacterium is defective with respect to UvrA, UvrB, or

both UvrA and UvrB. For instance, in some embodiments, the bacterium comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene. In some embodiments, the bacterium is defective with respect to SpoIIE, UvrA, and UvrB. In some embodiments, the bacterium is defective with respect to UvrC (e.g., the strain comprises a mutation in the *uvrC* gene). For instance, in some embodiments, the bacterium is defective with respect to RecA. In some embodiments, the bacterium comprises a mutation in the *recA* gene (e.g., comprises a temperature sensitive *recA* gene). In some embodiments, the bacterium comprises a *recA* gene that is under the control of the lac repressor. In some embodiments, the bacterium is a repressible *recA* mutant. In some embodiments, the bacterium expresses protective antigen (PA) under the control of an SOS regulatory sequence. In some embodiments, the bacterium comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacterium are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising the bacterium are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising the bacterium.

[0027] In another aspect, the invention provides a sporulation-deficient *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation. In some embodiments, the bacterium has been modified with a nucleic acid targeted compound that reacts directly with the nucleic acid. In some embodiments, the nucleic-acid targeted compound is a nucleic acid alkylator. In some embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., a psoralen compound activated by UVA irradiation). In some embodiments, the bacterium comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. In some embodiments, the bacterium is defective with respect to SpoIIE. In some embodiments, the bacterium comprises a mutation in a sporulation gene, such as, but not limited to, *spoIIE*. In some embodiments, the bacterium is attenuated for nucleic acid repair. For instance, in some embodiments, the bacterium is defective with respect to at least one DNA repair enzyme (e.g., UvrA, UvrB, and/or UvrC). In some embodiments, the bacterium comprises a mutation in one or more genes selected from the group consisting of *uvrA*, *uvrB*, and *uvrC*. In some embodiments, the bacterium is attenuated for recombinational repair.

For instance, in some embodiments, the bacterium is defective with respect to RecA. In some embodiments, the bacterium comprises a mutation in the *recA* gene. For instance, in some embodiments, the bacterium comprises a mutant *recA* gene which encodes a temperature-sensitive RecA. In some embodiments, the bacterium is a repressible or inducible *recA* mutant. In some embodiments, the bacterium expresses protective antigen (PA) under the control of an SOS regulatory sequence. In some embodiments, the bacterium comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. The invention further provides a vaccine or composition comprising the bacterium. In addition, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising the bacterium.

[0028] In still another aspect, the invention provides a *Bacillus anthracis* strain which is defective with respect to SpoIIE. In some embodiments, the strain is sporulation-deficient. In other embodiments, the strain is asporogenic. In some embodiments, the strain comprises a mutation in SpoIIE. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0029] In a further aspect, the invention provides a sporulation-deficient *Bacillus anthracis* strain that is attenuated for nucleic acid repair. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0030] In another aspect, the invention provides a asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair. Compositions, such as immunogenic compositions and vaccine compositions, comprising the bacteria of the strain are provided. Methods of inducing an immune response in a host comprising administering an effective amount of a composition

comprising a bacterium from the strain are also provided, as are methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain.

[0031] In some embodiments of each of the aforementioned aspects, as well as other aspects described herein, the strain and/or bacterium is isolated.

[0032] Methods of making and using the aforementioned strains and modified bacteria, as well as the other strains described herein are also provided.

[0033] Polynucleotides, expression cassettes, and vectors are provided as are host cells comprising the polynucleotides, expression cassettes, and vectors.

DRAWINGS

[0034] Figure 1 shows the attenuation of *Bacillus anthracis* Sterne strain with and without deletion of *uvrAB*. The log titer is plotted vs. nM concentration of psoralen S-59 present during growth and UVA irradiation (6 J/cm^2).

[0035] Figure 2 shows inactivation of various *B. anthracis* strains versus concentration of psoralen (S-59). Inactivation was measured by colony forming units (CFU). The S-59 concentrations were 0-2000 nM. The indicated *B. anthracis* strains were exposed to the indicated concentration of S-59, then treated with ultraviolet light (6.5 J/cm^2), followed by dilution and plating. The exposure time to the UV light was limited to about one minute. The *B. anthracis* strains were Sterne (-◇-)(open diamond); new Sterne (-□-)(open large square); SpoIIE (-△-)(open triangle); uvrAB (-▫-)(open small square); and uvrAB/SpoIIE (-O-) (open circle). New Sterne" was derived from the same bacterial stock as "Sterne," that is, the bacterium of "new Sterne" is identical to the bacterium of "Sterne," but they were derived from the same stock at different times.

[0036] Figure 3 shows colony forming units of *B. anthracis* before and after heating. The following strains of *B. anthracis* were tested: Sterne; uvrAB mutant; spoIIE mutant, spoIIE/uvrAB/cyaA mutant, and SpoIIE/uvrAB mutant.

DETAILED DESCRIPTION OF THE INVENTION

[0037] The present invention involves modified *Bacillus anthracis* strains and the use of the modified *Bacillus anthracis* in compositions, especially vaccine compositions. Modified *Bacillus anthracis* strains are provided which are attenuated for nucleic acid repair. Modified strains are also provided which are asporogenic (or sporulation-deficient), less toxic, and/or more immunogenic than the non-modified strains. In addition, modified *Bacillus anthracis* strains are provided which are modified so that proliferation of the strain is attenuated. Methods of both making and using the vaccine compositions are also provided.

[0038] The terms "modified" or "modification" as used herein with respect to strains and bacteria are intended to encompass such modification as chemical, physical, and genetic modification. For instance, in some embodiments, the bacteria are modified by a genetic mutation which attenuates the bacteria's ability to repair its nucleic acid. In some embodiments, a bacterium is modified by cross-linking of its genomic DNA with a psoralen activated by UVA radiation.

[0039] The inventors have engineered an attenuated strain of *Bacillus anthracis* which is particularly sensitive to inactivation by psoralens, a group of compounds that form irreversible cross-links in the genomes of bacteria after illumination with ultraviolet A (UVA) light, so that they are non-viable (see Examples 2 and 3, below). In addition, the inventors describe the construction of an asporogenic strain of *B. anthracis* (see Example 4, below) and a temperature sensitive *recA* mutant of *B. anthracis* (see Example 5, below). The introduction of mutations into the *B. anthracis* antigens Lef and Cya for decreased toxicity is also described (Example 6). The construction of a modified *B. anthracis* strain that expresses protective antigen under the control of SOS regulatory sequences is also provided (Example 7). This modified strain of *Bacillus anthracis* is designed to express protective antigen at high levels in the presence of DNA modification induced by an agent such as psoralen for enhanced immunogenicity. (Psoralen is also optionally used to attenuate the *B. anthracis* for proliferation.)

[0040] Accordingly, in one aspect, the present invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleotide excision repair (NER). In some embodiments, the strain comprises strain comprises an inhibiting mutation in, or a modification that attenuates expression of, at least one sporulation gene. In some embodiments, the strain is

defective with respect to SpoIIE. In some embodiments, the strain comprises a mutation in the *spoIIE* gene. Moreover, in some embodiments, the strain is defective with respect to one or more enzymes selected from the group consisting of UvrA, UvrB, and UvrC. In some embodiments, the strain comprises a mutation in at least one DNA nucleotide excision repair gene. For instance, in some embodiments, the strain comprises a mutation in one or more genes selected from the group consisting of *uvrA* gene, *uvrB* gene, and *uvrC* gene. In some embodiments, the strain further comprises an inhibiting mutation in, or a modification that attenuates expression of, a recombinational repair gene, such as *recA*. In some embodiments, the mutation or modification comprises a conditional mutation or a transcriptional or translational activator or repressor. In some embodiments, the mutation comprises a conditional mutation. In some embodiments, the modification comprises a nucleic acid molecule encoding a RecA protein under the control of a transcriptional or translational activator or repressor (e.g., the lac repressor). In some embodiments, the strain comprises strain further comprising an inhibiting mutation, or a modification that attenuates expression of, at least one toxin gene. In some embodiments, the at least one toxin gene comprises the *lef* gene, *cya* gene, or *lef* and *cya* genes. In some embodiments, the strain further comprises a nucleic acid encoding at least one antigen, wherein the nucleic acid encoding the at least one antigen is operably linked to a heterologous promoter. In some embodiments, the heterologous promoter is inducible. For instance, the promoter is, in some embodiments, inducible by ultraviolet light, a nucleic acid targeted compound, a nucleic acid cross-linking compound, and/or an SOS regulatory pathway. In some embodiments, the nucleic acid encoding the at least one antigen is or is derived from: *pagA* gene; *lef* gene; *cya* gene; *pagA* and *lef* genes; *pagA* and *cya* genes; *cya* and *lef* genes; or *pagA*, *lef*, and *cya* genes. In some embodiments, the strain comprises a poly-D-glutamate capsule, while in some alternative embodiments the strain lacks a poly-D-glutamate capsule. A bacterium of the strain is also provided which further comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. A vaccine or composition comprising the *Bacillus anthracis* strain is also provided, as well as a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to a host an effective amount of a composition comprising a bacterium of the strain.

[0041] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a nucleic acid encoding an antigen, wherein the nucleic acid encoding the antigen is operably linked to a heterologous promoter. In some embodiments, the nucleic acid encoding the antigen is, or is derived from: *pagA* gene; *lef* gene; *cya* gene; *pagA* and *lef* genes; *pagA* and *cya* genes; *cya* and *lef* genes; or *pagA*, *lef*, and *cya* genes. In some embodiments, the heterologous promoter is inducible. In some embodiments, the heterologous promoter is inducible by: ultraviolet light, a nucleic acid cross-linking compound, ultraviolet light and a nucleic acid cross-linking compound, an SOS regulatory pathway, and/or a change or shift in temperature. The invention further provides a vaccine or composition comprising a bacterium of the *Bacillus anthracis* strain. The present invention also provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to a host an effective amount of a composition comprising a bacterium of the strain.

[0042] In an additional aspect, the invention provides a *Bacillus anthracis* strain comprising at least one inhibiting mutation in each of *uvrA* gene, *uvrB* gene, *lef* gene, *cya* gene, and *spoIIE* gene. Also provided is a bacterium of the *Bacillus anthracis* strain, wherein the bacterium further comprises at least one covalently linked nucleic acid cross-linking compound linked to the genomic DNA of the bacterium.

[0043] In another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair. In one embodiment, the strain is defective with respect to SpoIIE. In some embodiments the strain is also defective with respect to UvrA, UvrB, and/or UvrC (e.g., comprises a mutation in the *uvrAB* gene). In some embodiments the strain is also defective with respect to RecA. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0044] In another aspect, the invention provides a *Bacillus anthracis* strain comprising a heterologous expression cassette comprising a nucleic acid sequence encoding protective antigen, wherein the nucleic acid sequence encoding protective antigen is operably linked to an inducible promoter. In some embodiments, the expression of the protective antigen is induced by treatment of the strain with a psoralen and UVA radiation. For instance, in some embodiments, the protective antigen is operably linked to an SOS regulatory sequence, or any promoter that is induced in response to photochemical treatment or other modification of the DNA of the bacterium. In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, at least one nucleic acid repair gene (e.g., a nucleotide excision repair (NER) gene, a recombinational repair gene, or an NER gene and a recombinational repair gene). In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, the nucleic acid repair gene is selected from the group consisting of *uvrA*; *uvrB*; *uvrC*; *uvrA* and *uvrB*; and *uvrA*, *uvrB*, and *uvrC*. In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, *recA*. In some embodiments, the mutation or modification comprises a conditional mutation or a transcriptional or translational activator or repressor. In some embodiments, the mutation comprises a conditional mutation. In some embodiments, the modification comprises a nucleic acid molecule encoding a RecA protein under the control of a transcriptional or translational activator or repressor. In some embodiments, the strain comprises a mutation that attenuates the ability of the strain to repair its nucleic acid. For instance, in some embodiments, the strain is defective with respect to at least one DNA repair enzyme, such as UvrA, UvrB, or both UvrA and UvrB. In some embodiments, the strain comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene. In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments, the strain is defective with respect to RecA. A strain that is defective with respect to RecA can, by way of example, comprise a mutation in the *recA* gene, comprise a *recA* gene under control of a repressible promoter, or comprise a temperature sensitive *recA* gene. In some embodiments, the strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the bacteria of the strain further comprise at least one covalently linked

nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacteria. In addition, the strain is, in some embodiments, asporogenic (e.g., a *spoIIE* mutant). In some embodiments, the strain comprises an inhibiting mutation in, or a modification that attenuates expression of, at least one sporulation gene or toxin gene (e.g., *spoIIE* gene; *lef* gene; *cya* gene; *spoIIE* and *lef* genes; *spoIIE* and *cya* genes; *lef* and *cya* genes; *spoIIE*, *lef*, and *cya* genes). In some embodiments, the strain comprises a poly-D-glutamate capsule, whereas in other embodiments, the strain lacks a poly-D-glutamate capsule. Vaccines and compositions comprising the strain are also provided. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of the strain is also provided. A method of protecting a host from a disease, comprising administering to the host an effective amount of a composition (e.g., a vaccine) comprising a bacterium from the strain is also provided.

[0045] The invention also provides a *Bacillus anthracis* strain comprising a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the *Bacillus anthracis* strain is attenuated for nucleic acid repair (e.g., is defective for UvrA, UvrB, UvrC, and/or RecA). In some embodiments, the strain is asporogenic. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host to Bacilllus *anthracis* comprising administering to the host an effective amount of the composition also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0046] In yet another aspect, the invention provides an *Bacillus anthracis* strain that is defective with respect to RecA. In some embodiments, the strain is a conditional *recA* mutant. In some embodiments, the strain is a temperature sensitive *recA* mutant. In some embodiments, the strain is a repressible *recA* mutant. In some embodiments, the strain comprises a heterologous expression cassette encoding protective antigen, wherein the protective antigen is operably linked to an SOS regulatory sequence. In some embodiments is defective with respect to at least

one additional DNA repair enzyme such as UvrA, UvrB, or both UvrA and UvrB. (For instance, the strain optionally comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene.) In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments, the modified strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the *Bacillus anthracis* strain is asporogenic (e.g., defective with respect to SpoIIE). Vaccine compositions comprising a bacterium from the *Bacillus anthracis* strain are also provided. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium from the strain is also provided. A method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium from the strain is further provided.

[0047] The invention further provides a *Bacillus anthracis* strain comprising a temperature sensitive *recA* gene. In some embodiments, the strain is defective with respect to UvrA and/or UvrB. In some embodiments, the strain is defective with respect to UvrC. In some embodiments, the strain is asporogenic. In some embodiments, the strain comprises a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response to *Bacillus anthracis* in a host comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0048] In another aspect, the invention provides a *Bacillus anthracis* bacterium comprising a mutation in its *recA* gene, wherein the *recA* gene encodes a temperature-sensitive RecA protein comprising a V244M mutation. Vaccines and compositions comprising the bacterium are provided as are methods of inducing an immune response in a host to *Bacillus*

anthracis comprising administering to the host an effective amount of a composition comprising the bacterium.

[0049] In a further aspect, the invention provides a *Bacillus anthracis* bacterium comprising a *recA* gene derived from a foreign bacterium, wherein the *recA* gene encodes a temperature-sensitive RecA protein. In some embodiments, the *recA* gene is derived from *E. coli*, wherein the *recA* gene encodes a temperature-sensitive RecA protein comprising a V246M mutation. Vaccines and compositions comprising the bacterium are provided as are methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising the bacterium.

[0050] In another aspect, the invention provides a *Bacillus anthracis* bacterium which is a repressible or inducible *recA* mutant. In some embodiments, the *recA* gene is under control of a lac repressor. Vaccines and compositions comprising the bacterium are provided as are methods of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising the bacterium.

[0051] In another aspect, the invention provides an *Bacillus anthracis* strain attenuated for nucleic acid repair, wherein the strain is defective with respect to RecA and at least one additional DNA repair enzyme selected from the group consisting of UvrA, UvrB, and UvrC. In some embodiments, the strain defective with respect to RecA is also defective with respect to both UvrA and UvrB. Compositions, such as vaccine compositions, comprising the bacterium of the strain are also provided. Methods of using compositions comprising the strain are also provided.

[0052] In another aspect, the invention provides an asporogenic *Bacillus anthracis* strain attenuated for nucleic acid repair, wherein the strain is defective with respect to SpoIIE and at least one additional DNA repair enzyme selected from the group consisting of UvrA, UvrB, and UvrC. In some embodiments, the asporogenic strain defective with respect to SpoIIE is also defective with respect to both UvrA and UvrB. Compositions, such as vaccine compositions, comprising the bacterium of the strain is also provided. Methods of using compositions comprising the strain are also provided.

[0053] In still another aspect, the invention provides an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair, comprising a temperature sensitive *recA* gene and a mutation in the *uvrAB* gene. Compositions, such as vaccine compositions, comprising the

bacterium of the strain is also provided. Methods of using compositions comprising the strain are also provided.

[0054] In another aspect, the invention provides an asporogenic *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation. In some embodiments, the bacterium has been modified with a nucleic acid targeted compound that reacts directly with the nucleic acid. In some embodiements, the nucleic-acid targeted compound is a nucleic acid alkylator. In some embodiments, the nucleic acid targeted compound is a psoralen compound (e.g., a psoralen compound activated by UVA irradiation). In some embodiments, the bacterium comprises at least one covalently linked nucleic acid cross-linking compound (e.g., a psoralen), linked to the genomic DNA of the bacterium. In some embodiments, the bacterium is defective with respect to SpoIIE. In some embodiments, the bacterium comprises a mutation in a sporulation gene, such as, but not limited to, spoIIE. In some embodiments, the bacterium is attenuated for nucleic acid repair. For instance, in some embodiments, the bacterium is defective with respect to at least one DNA repair enzyme (e.g., UvrA, UvrB, and/or UvrC). In some embodiments, the bacterium comprises a mutation in one or more genes selected from the group consisting of *uvrA*, *uvrB*, and *uvrC*. In some embodiments, the bacterium is attenuated for recombinational repair. For instance, in some embodiments, the bacterium is defective with respect to RecA. In some embodiments, the bacterium comprises a mutation in the *recA* gene. For instance, in some embodiments, the bacterium comprises a mutant *recA* gene which encodes a temperature-sensitive RecA. In some embodiments, the bacterium is a repressible or inducible *recA* mutant. In some embodiments, the bacterium expresses protective antigen (PA) under the control of an SOS regulatory sequence. In some embodiments, the bacterium comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. The invention further provides a vaccine or composition comprising the bacterium. In addition, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising the bacterium.

[0055] As used herein, including the appended claims, the singular forms of words such as "a," "an," and "the" include their corresponding plural references unless the context clearly dictates otherwise.

[0056] Where aspects or embodiments of the invention are described in terms of a Markush group or other grouping of alternatives, the present invention encompasses not only the entire group listed as a whole, but each member of the group individually and all possible subgroups of the main group, but also the main group absent one or more of the group members. The present invention also envisages the explicit exclusion of one or more of any of the group members in the claimed invention.

I. MODIFIED *BACILLUS ANTHRACIS* BACTERIA

[0057] In some embodiments, the modified *Bacillus anthracis* strain is derived from a strain selected from the group consisiting of Ames, Vollum, A1.a/10, A1.b/23, A2/29, A3.a/34, A3.b/57, A4/69, B/80, Δsterne, VN41Δ1, Dames, NNR1Δ1, and DNH1. In some embodiments, the *Bacillus anthracis* strain is derived from the Ames strain or the Sterne strain. For instance, in some embodiments, the modified strain of *Bacillus anthracis* is derived from the Sterne strain. In other embodiments, the modified strain of *Bacillus anthracis* is derived from the Ames strain.

[0058] In some embodiments, the modified strains are isolated. A strain, bacterium, or composition which is "isolated" is a strain, bacterium, or composition, which is in a form not found in nature. Isolated strains, bacteria, or compositions include those which have been purified to a degree that they are no longer in a form in which they are found in nature. In some, but not all, embodiments, a strain, bacterium, or composition which is isolated is substantially pure.

[0059] In some embodiments, the *Bacillus anthracis* comprises one or more virulence plasmids. In some embodiments, the virulence plasmid comprises the native sequence. In other embodiments, the virulence plasmid is a recombinant plasmid. In some embodiments, the *Bacillus anthracis* strain comprises a pXO1 plasmid. In some other embodiments, the *Bacillus anthracis* strain comprises a pXO2 plasmid. In some embodiments, the *Bacillus anthracis* strain comprises both a pXO1 plasmid and a pXO2 plasmid (i.e., pXO1+ and pXO2+). In other embodiments, the strain lacks a pXO1 plasmid and/or the pXO2 plasmid. In some other embodiments, the strain is pXO1- and pXO2 +. In some embodiments, the strain is pXO1+ and pXO2-. In still other embodiments, the strain is pXO1- and pXO2-.

[0060] *B. anthracis* can include a capsule, where the capsule comprises poly-gamma-D-glutamate (also referred to as a poly-D-glutamate capsule). The capsule has an

anti-phagocytic role, promotes virulence, and has immunological properties. Intracellular host conditions stimulate biosynthesis of the capsule. The poly-gamma-D-glutamate is encoded by a large plasmid (pXO2) residing in the bacterium (see, e.g., Mesnage, et al. (1998) J. Bacteriol. 180:52-58; Fouet and Mesnage (2002) Curr. Top. Microbiol. Immunol. 271:87-113; Green, et al. (1985) Infection Immunity 49:291-297).

[0061] In some embodiments, the modified strain comprises a capsule encoding plasmid, such as a pXO2 plasmid which encodes a poly-D-glutamate capsule. In other embodiments, the strain does not comprise a capsule-encoding plasmid or is otherwise defective in production of the poly-D-glutamate capsule.

A. *Bacillus anthracis* bacteria modified by mutations and/or heterologous polynucleotides

[0062] In some embodiments, the modified *Bacillus anthracis* strain of the invention has been modified by mutation. The mutation may be a mutation of any type. For instance, the mutation may constitute a point mutation, a frame-shift mutation, an insertion, a deletion of part or all of a gene. In addition, in some embodiments of the modified strains, a portion of the *B. anthracis* genome has been replaced with a heterologous polynucleotide. In some embodiments, the modification comprises insertion of a heterologous polynucleotide into the genomic DNA of *B. anthracis*. In some embodiments, the mutations are naturally-occurring. In other embodiments, the mutations are the results of artificial mutation pressure. In still other embodiments, the mutations in the *B. anthracis* genome are the result of genetic engineering. For safety reasons, in some embodiments, it may be preferred that the mutation be a double mutation or a deletion mutant rather than a point mutation. A mutant in which a gene "*xyz*" has been deleted is alternatively referred to herein as Δ*xyz*, *delta*xyz, *xyz*$^-$, or an *xyz* deletion mutant. For instance, a bacterial strain in which the uvrA gene has been deleted is alternatively referred to herein as uvrA mutant, Δ*uvrA,* or *uvrA*$^-$. In addition, it will be understood by one of ordinary skill in the art that a reference to a particular mutant or strain as an "*xyz*" mutant or "*xyz*" strain will sometimes refer to a mutant or strain in which the *xyz* gene has been deleted. In some embodiments, the *Bacillus anthracis* strains have been modified by introduction of a heterologous polynucleotide, such as a vector, plasmid, and/or expression cassette into the bacteria. Although in some embodiments, the heterologous polynucleotide may be integrated

into the genome, in other embodiments, the bacteria comprise a heterologous polynucleotide which is not integrated.

[0063] In some embodiments, a modified strain is attenuated for nucleic acid repair; attenuated for sporulation; less toxic; and/or more immunogenic (relative to wild-type or the same strain without the modification).

[0064] In some embodiments, the modified bacteria are defective with respect to one or more nucleic acid repair enzymes (e.g., a nucleotide excision repair enzyme or a recombinational repair enzyme), one or more sporulation proteins, or one or more toxins (relative to wild-type or the same bacteria without the modification). For instance, in some embodiments, the modified bacteria are defective with respect to one or more of the following proteins: UvrA, UvrB, UvrC, RecA, SpoIIE, lethal factor, and edema factor. In some embodiments, the modified strains are defective with respect to a protein due to a mutation in the gene encoding the protein or a modification that attenuates expression of the gene encoding the protein (for instance, due to the introduction of heterologous nucleic acid molecules into the *Bacillus anthracis*, such as antisense expression cassettes).

[0065] In some embodiments, the modified bacteria comprise a mutation in one or more genes selected from the group consisting of nucleic acid repair genes (e.g., nucleotide excision repair genes and recombinational repair genes), sporulation genes, and toxin genes. For instance, in some embodiments, the modified bacteria comprise a mutation in one or more genes selected from the group consisting of *uvrA*, *uvrB*, *uvrC*, *recA*, *spoIIE*, *lef*, and *cya*.

[0066] In some embodiments, the mutations in the *Bacillus anthracis* strains described herein are inhibiting mutations.

[0067] An "inhibiting mutation" of a nucleic acid encompasses, e.g., deletion mutations, frameshift mutations, point mutations, and/or insertion mutations, where the functional result of the mutation comprises an inhibition of one or more functions of the gene. For example, an inhibiting mutation in a gene encoding a nucleotide excision repair gene can be one that results in a reduction in rate of formation of the repair complex, in a reduction in rate of binding of the repair complex to DNA, in a reduction in rate of DNA incision efficiency, in an increase in frequency of damaged DNA lesions in the genome, in an increase in stable mutations arising from said increase in frequency, and/or an increase in cell death.

[0068] In some embodiments, expression of one or more genes in the modified bacteria is attenuated (relative to wild-type or the same bacteria without the modification). In some embodiments, the one or more genes whose expression is attenuated are selected from the group consisting of nucleic acid repair genes (e.g., nucleotide excision repair genes and recombinational repair genes), sporulation genes, and toxin genes. For instance, in some embodiments, the modified bacteria are attenuated in expression of one or more genes selected from the group consisting of *uvrA*, *uvrB*, *uvrC*, *recA*, *spoIIE*, *lef*, and *cya*.

[0069] A "modification that attenuates expression of a gene" or a "modification attenuating expression of a gene" in a bacterium encompasses a mutation or other modification, such as introduction of a heterologous nucleic acid molecule into the bacterium, wherein the mutation or other modification in the bacterium results in a decrease in the expression of the specified gene. The decrease in the expression of the gene can be, but is not limited to, a decrease in the transcription of the gene and/or a decrease in the translation of the gene. Thus, a modification that attenuates expression of a gene embraces reagents and methods wherein translation of the gene is reduced by an antisense nucleic acid.

[0070] The present invention contemplates reagents and methods using antisense nucleic acid technology, e.g., antisense RNA or antisense DNA. This includes, but is not limited to, the use of a genomic polynucleotide or plasmid-based polynucleotide encoding the antisense nucleic acid, wherein the antisense nucleic acid inhibits translation of a *B. anthracis* nucleic acid (see, e.g., Wagner and Simons (1994) Ann. Rev. Microbiol. 48:713-742; Johansson and Cossart (2003) Trends Microbiol. 11:280-285; Wang and Kuramitsu (2003) FEMS Microbiol. Lett. 220:171-176; Lipman (1997) Nucleic Acids Res. 25:3580-3583; Asano, et al. (1998) J. Biol. Chem. 273:11826-11838; Mao, et al. (1995) J. Biol. Chem. 270:19684-19687). In some embodiments, the modification that attenuates expression of a gene in the *Bacillus anthracis* is a heterologous antisense expression cassette encoding an RNA complementary to a portion of an RNA product (e.g., mRNA) of the gene and able to hybridize to the RNA product under cellular conditions.

[0071] In some embodiments, the present invention embraces *B. anthracis* strains and methods wherein expression of a sporulation gene such as the *spoIIE* gene, a toxin gene such as the *lef* gene or *cya* gene, and/or a nucleic acid repair gene such as a nucleotide excision repair (NER) gene or a recombinational repair gene, and/or activity of the polypeptide expressed from

said gene or genes, is reduced or inhibited by at least about 10%, by at least about 20%, by at least about 30%, by at least about 40%, by at least about 50%, by at least about 60%, by at least about 70%, by at least about 80%, by at least about 90%, by at least about 95%, by at least about 99%, or by at least about 99.9% (relative to wild-type or the non-modified strain).

[0072] In some embodiments, the *B. anthracis* strain is attenuated for nucleic acid repair. In some embodiments, the *B. anthracis* contains a mutation (i.e., a genetic mutation) that reduces (preferably, significantly) the ability of the bacteria to repair modifications to their nucleic acid, including, but not limited to, adducts occurring on pyrimidine residues within the bacterial genome resulting from modification by nucleic acid modifying agents, such as, for example, psoralens, 4-nitroquinoline oxide, cisplatin, mitomycin C, or benzo[a]pyrene. Such a mutation (also referred to herein as a "genetic mutation") could be in any of a variety of genes that are involved in the DNA repair mechanisms of the bacteria (Aravind et al., Nucleic Acids Research 27(5):1223-1242 (1999)). Bacteria that are deficient in their ability to repair damage to their nucleic acid provide an added level of safety and efficacy to the use of the bacteria of the present invention. In particular, using the appropriate repair deficient mutants, the bacteria are exquisitely sensitive to nucleic acid modification (see Section I.B, below). The nucleic acid of the bacteria may be modified to a lesser degree than for the non-mutant bacteria, yet still ensure the desired amount of attenuation of proliferation. This provides a larger window of efficacy in which to operate so that the expression of the bacterial nucleic acid is sufficient to generate the desired antigenic proteins and/or other factors required to induce a desired immune response *de novo* in an animal immunized with designated vaccine compositions. The larger window of efficacy results from a random distribution of infrequent DNA modifications in the mutant bacteria compared to the non-mutant bacteria, and as a result, the expression of desired antigenic proteins and/or other necessary factors, within a *B. anthracis* population comprising a vaccine dose is not affected or is substantially less affected. It also provides an added level of safety as the level of attenuation of proliferation achieved cannot be compromised by repair of the modified nucleic acid. In another embodiment, the genetic mutation alters the susceptibility of the bacteria to treatment with a nucleic acid targeted compound, for example by altering the permeability of the bacteria to the compound or by altering the ability of the compound to access and bind the bacterial nucleic acid. Such mutations may also impact the efficacy of the process of attenuating proliferation while leaving bacterial gene expression substantially unaffected.

[0073] In some embodiments, the modified *Bacillus anthracis* strain or bacterium is attenuated for nucleic acid repair (e.g., nucleotide excision repair and/or recombinational repair), relative to wild-type or the non-modified strain or bacterium. In some embodiments, the ability of the modified strain or bacterium to repair its nucleic acid by one or more nucleic acid repair pathways is at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 90%, at least about 95%, or at least about 98%, reduced relative to wild-type or a non-modified strain or bacterium. In some embodiments, the ability of the modified strain or bacterium to repair a modification made to its nucleic acid in order to attenuate the strain or bacterium for proliferation, by one or more nucleic acid repair pathways relevant to the modification, is at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 90%, at least about 95%, or at least about 98%, reduced relative to wild-type or a non-modified strain or bacterium. In some embodiments, the bacterium or strain is attenuated for nucleic acid repair due to a mutation. In some other embodiments, the bacterium or strain is attenuated because expression of one or more genes is reduced. In some embodiments, the bacterium or strain is attenuated with respect to nucleic acid repair (relative to wild-type or a non-modified bacterium) only under certain conditions (e.g., a certain temperature or pH or the presence or absence of certain agents, such as IPTG, which induce or repress expression).

[0074] In some embodiments, the modified strain or bacterium is defective with respect to at least one DNA repair enzyme (relative to wild-type or the non-modified strain or bacterium). In some embodiments, the activity level of a DNA repair enzyme in a *Bacillus anthracis* strain or bacterium which is defective with respect to that DNA repair enzyme is decreased by at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 90%, at least about 95%, or at least about 98%, relative to wild-type or a non-modified strain or bacterium. In some embodiments, the bacterium or strain is defective with respect to a DNA repair enzyme because the bacterium or strain does not express the DNA repair enzyme or shows decreased expression (or conditional expression) of the DNA repair enzyme. In some other embodiments, the bacterium or strain is defective with respect to a DNA repair enzyme because the bacterium or strain expresses a mutant form of the DNA repair enzyme, that is less active or is conditionally active. In some embodiments, the bacterium or strain that is defective with respect to a DNA repair enzyme because the bacterium or strain is conditionally defective with respect the enzyme. For instance, in some embodiments, the bacterium or strain

may only be defective with respect to the DNA repair enzyme (relative to wild-type or a non-modified bacterium) under certain conditions (e.g., a certain temperature or pH or the presence or absence of certain agents, such as IPTG).

[0075] To illustrate the advantages of using a repair deficient mutant, one can consider the mechanism of the attenuation of bacterial proliferation (see Section I.B, below). The bacterial nucleic acid is modified either by strand breakage or pyrimidine dimers, or by chemical modifications such as monoadducts or crosslinks. If the mechanisms for repair of these modifications are intact, a certain number of modifications will be required in order to overcome the bacterial DNA repair mechanisms, and achieve sufficient attenuation of proliferation. The greater the modification of nucleic acid, the greater the reduction in protein expression, and the greater the reduction of protein expression of any given gene, within a bacterial population comprising a vaccine dose. Even though the levels of modification required to attenuate proliferation are much lower than the levels required to stop protein expression, protein expression will still be reduced to some extent, possibly to a level unacceptable to generate a desired immune response in a vaccinated individual. The use of DNA repair deficient mutants significantly reduces the levels of nucleic acid modification needed to attenuate proliferation such that a lower modification level will result in adequate attenuation of proliferation. Since the nucleic acid modification is much lower, the expression of proteins will be less affected, providing for a higher level of expression of the protein(s) of interest. Such repair deficient mutants are particularly useful in the preparation of vaccines, where the safety of the vaccine can be increased by a slight modification of the nucleic acid, leaving a sufficiently high level of protein expression, in particular of the antigen to which the immune response is targeted.

[0076] In one embodiment the repair deficient mutant is unable to repair interstand crosslinks. Such mutants include, but are not limited to, mutations in *uvr* genes, i.e. *uvrA*, *uvrB*, *uvrC*, and *uvrD* genes as well as *rec*A genes. The mutations may be in one or more of these genes. These mutations result in attenuation in the activity of the corresponding enzymes UvrA (an ATPase), UvrB (a helicase), UvrC (a nuclease), UvrD (a helicase II) and RecA (a recombinase). These mutants would most typically be used in conjunction with a crosslinking compound, such as a psoralen. (See, e.g., Section I.B, below.) Since the bacterial nucleic acid is crosslinked in some locations, and these crosslinks cannot be repaired, the bacteria are unable to replicate as the original strands of nucleic acid can not be separated. Since they cannot be

repaired, very few crosslinks are needed, the bacterial nucleic acid is for the most part accessible for transcription, and protein expression is not altered significantly. In some embodiments, a population of repair deficient bacterial mutants that are unable to repair interstrand crosslinks are suitably crosslinked such that essentially every bacterium in the population contains at least one crosslink, such that attenuation of replication is essentially complete, wherein the bacterial gene expression of the population is sufficiently active.

[0077] In some embodiments, the *B. anthracis* strain comprises an inhibiting mutation in or a modification that attenuates expression of at least one nucleic acid repair gene. The nucleic acid repair gene is, e.g., a nucleic acid excision repair gene or a recombinational repair gene. Nucleic acid repair, mediated by a number of individual enzymes and protein complexes, includes repair by nucleotide excision repair (NER) and recombinational repair. Nucleic acid repair enzymes, protein complexes, and pathways have been characterized in *B. anthracis*, *B. subtilis*, *E. coli*, and in other organisms. In *E. coli*, the NER pathway begins with the uvrABC endonuclease which comprises a complex of UvrA, UvrB, and UvrC proteins. The role of proteins such as RecA, RecF, and RecR, in repairing DNA damage has been described. Expression of the excision repair genes *uvrA*, *uvrB*, and *uvrD*, but not *uvrC*, are upregulated as part of the SOS response. In some embodiments, the strain further comprises an inhibiting mutation in or a modification that attenuates expression of another gene, e.g., *spoIIE* gene, *lef* gene, *cya* gene, or any combination thereof.

[0078] In some embodiments, the modified *Bacillus anthracis* strain is attenuated for nucleotide excision repair. For instance, in some embodiments, the strain is defective with respect to a nucleotide excision repair enzyme. In some embodiments, the strain comprises a inhibiting mutation in, or a modification that attenuates expression of one or more nucleotide excision repair genes (e.g., UvrA, UvrB, UvrC, and /or UvrD). In some embodiments the strain comprises a mutation in a nucleotide excision repair gene.

[0079] In some embodiments, the modified *Bacillus anthracis* strain is attenuated for recombinational repair. For instance, in some embodiments, the bacteria are defective with respect to a recombination repair enzyme. In some embodiments, the bacteria of the strain comprise an inhibiting mutation in, or a modification that attenuates expression of, a recombination repair enzyme, such as, but not limited to, RecA. The inhibited, mutated, or attenuated recombinational repair genes of the present invention encompass, but are not limited

to, *recA*, *recD*, *recF*, *recG*,or *ruvABC* (Munakata, et al. (1991) Photochem. Photobiol. 54:761-768; Carrasco, et al. (2002) Mol. Genet. Genomics 266:899-906; Alonso and Luder (1991) Biochimie 73:277-280; Kuzminov (1999) Microbiol. Mol. Biol. Rev. 63:751-813). In some embodiments, the recombinational repair gene is *recN*. In other embodiments, the bacteria or strain that is defective with respect to a DNA repair enzyme and/or a recombination repair enzyme is not defective with respect to RecN. (The terms "inhibited," "mutated," and "attenuated" are not necessarily exclusive of each other, and can coexist, when describing, e.g., a reagent, molecule, nucleic acid, gene, enzyme, protein, or bacterium.)

[0080] In some embodiments, the modification that attenuates expression of a recombination repair enzyme (e.g., *recA*) places expression of the recombination repair enzyme under the control of a transcriptional activator or repressor. In some embodiments, the modification that attenuates expression of a recombination repair enzyme places expression of the recombination repair enzyme under the control of a translational activator or repressor. In some embodiments, the transcriptional repressor is the lac repressor.

[0081] In one embodiment the modified *Bacillus anthracis* strain is defective with respect to RecA. In some embodiments, the defective strain comprises a mutation in the *recA* gene. In some embodiments, the defective strain is a conditional *recA* mutant, such as a temperature sensitive *recA* mutant. In some embodiments, the defective strain is a repressible *recA* mutant. In some embodiments, the strain defective in RecA is an inducible *recA* mutant. In some embodiments, the strain that is defective with respect to RecA comprises a *recA* gene under the control of a transcriptional repressor. For instance, in some embodiments, a *recA* gene may be operably linked to a promoter and a regulatory sequence which binds a transcriptional repressor and the transcriptional repressor is encoded by an expression cassette introduced within the *B. anthracis*. In some alternative embodiments, two expression cassettes are introduced to the bacteria. One expression cassette comprises a polynucleotide encoding an anti-recA antisense RNA operably linked to a promoter and a regulatory sequence which binds the transcriptional repressor. The other expression cassette encodes the transcriptional repressor. In some embodiments, the transcriptional repressor is the lac repressor.

[0082] In some embodiments, a mutation (e.g., an inhibiting mutation) in the *recA* gene is a conditional mutation. In such a mutation, the mutation in the *recA* gene results in the attenuation in the activity of *recA* only under certain conditions (i.e. non-permissive conditions),

such as a suitable pH or temperature of the bacterial population. Bacteria comprising a conditional *recA* mutation can be cultured under permissive conditions in order to grow sufficient levels of the bacteria and then placed under non-permissive conditions for treatment to modify the nucleic acid, then optionally stored under non-permissive conditions such that the nucleic acid damage is not adequately repaired. As an example of this, a *recA* temperature sensitive mutant is grown at 30 °C, where it grows well, and is treated to modify the nucleic acid at 42 °C, which is non-permissive for *recA* such that it is very sensitive to treatment, such as psoralen crosslinking.

[0083] The present invention provides regulatory sequences sensitive to, e.g., temperature, pH, osmotic changes, or alterations in the concentrations of oxygen, ions, or metabolites for use in the nucleic acids, bacteria and methods of the present invention (see, e.g., Repoila and Gottesman (2003) J. Bacteriol. 185:6609-6614; Schofield, et al. (2003) Appl. Environ. Microbiol. 69:3385-3392; Hanna, et al. (2001) J. Bact. 183:5964-5973; Deuerling, et al. (1995) J. Bacteriol. 177:4105-4112). One or more of these regulatory sequences can be used to allow expression of *recA* during growth of *B. anthracis*, but to prevent or inhibit expression of recA before, or shortly before, or during exposure of the *B. anthracis* to reagents that inflict DNA damage. In the case of a temperature sensitive mutant, the location of the mutation need not necessarily be in a regulatory region; the mutation can be in the coding region of the gene. For example, a temperature sensitive *recA* mutant was produced by a mutation in the coding region of the *recA* gene (Kawashima et al. *Mol. Gen. Genet.* 193:288-92 (1984)).

[0084] In some embodiments, the *Bacillus anthracis* bacterium which is a temperature-sensitive *recA* mutant comprises a temperature-sensitive *recA* mutant gene. For instance, in some embodiments, the strain comprises a mutant *recA* gene which encodes a temperature-sensitive RecA protein. In some embodiments, the temperature-sensitive RecA is functional at temperatures suitable for growing the bacteria, but non-functional or less functional at higher temperatures which can be used during treatment with nucleic-acid targeted compounds and/or ultraviolet light when it is desirable to attenuate the ability of the bacteria for recombinational repair. In some embodiments, the temperature-sensitive *recA* mutant gene comprises a mutation analogous to that of the recA44 temperature sensitive allele of *E. coli* (V246M; Kawashima et al., Mol. Gen. Genet. 193:288-92 (1984); Hall et al., J. Bacteriol. 121:892-900 (1975)). In some embodiments, the bacterium or strain comprises a *recA* gene which encodes a temperature-

sensitive RecA protein comprising a mutation analogous to the V246M mutation of the recA44 temperature sensitive *recA* mutant of *E. coli*. For instance, in some embodiments, the modified *Bacillus anthracis* bacterium comprises a mutant *Bacillus anthracis recA* gene which encodes a temperature-sensitive mutant RecA protein comprising the mutation V244M (or a functional fragment or variant of such a protein). In some embodiments, the *Bacillus anthracis* bacterium comprises a polynucleotide comprising a polynucleotide encoding SEQ ID NO:54 (see sequence below), or a functional fragment or variant thereof.

[0085] In some alternative embodiments, the modified *B. anthracis* bacterium comprises a *recA* gene derived from a foreign bacterium, wherein the *recA* gene encodes a temperature-sensitive RecA protein. In some embodiments, the mutant *recA* gene is derived from *E. coli*. For instance, in some embodiments, the modified *Bacillus anthracis* bacterium comprises an *E. coli recA* gene which encodes a temperature-sensitive RecA protein, or a functional fragment thereof. For instance, in some embodiments, the modified *B. anthracis* bacterium (or strain) comprises a *recA* gene that comprises the recA44(ts) allele of *E. coli* . Alternatively, the modified *B. anthracis* comprises the coding sequence of the recA44(ts) allele operably linked to a promoter functional in *Bacillus anthracis*. In some embodiments, the *Bacillus anthracis* bacterium comprises a polynucleotide comprising a polynucleotide encoding SEQ ID NO:54 (see sequence below), or a functional fragment thereof. In some embodiments, the bacterium comprises an *E. coli recA* gene, wherein the *E. coli recA* gene encodes a temperature-sensitive RecA protein comprising a V246M mutation within the sequence 245KVVKNK250 (SEQ ID NO:14) or a V247M mutation within the sequence 245VKVVKNK251 (SEQ ID NO:56). In some embodiments, the *Bacillus anthracis* bacterium or strain comprises a nucleic acid encoding a temperature-sensitive RecA protein in which the sequence KVVKNK (SEQ ID NO:14) has been mutated to KMVKNK (SEQ ID NO:57).

[0086] In some embodiments, the bacteria comprise a nucleic acid molecule encoding a RecA protein, or functional fragment or variant thereof, which is a temperature-sensitive mutant.

[0087] It is possible that upon vaccination, the conditions may permit expression of *recA*, resulting in some repair and presenting a safety issue. In some embodiments, it may be preferable to use a mutation of the *recA* gene which is a double mutation or a deletion mutant for safety reasons.

[0088] The invention further provides modified *B. anthracis* strains and bacteria which are repressible or inducible *recA* mutants. In some embodiments, the bacteria are repressible mutants. In some embodiments, the bacteria are inducible mutants.

[0089] In some embodiments, the invention provides a *Bacillus anthracis* bacterium (and strain) that comprises a nucleic acid molecule encoding a RecA protein, wherein expression of the RecA protein is under the control of a transcriptional or translational activator or repressor (e.g., a transcriptional repressor, such as the lac repressor). In some embodiments, a RecA protein is expressed in the bacterium under the control of a heterologous transcriptional repressor or activator. In some embodiments, expression of the *recA* gene is under control of a lac repressor.

[0090] In some embodiments, the invention provides a *Bacillus anthracis* bacterium (and strain) that comprises a nucleic acid encoding a RecA protein, wherein the nucleic acid is operably linked to a heterologous operator which binds a repressor or activator. (Typically a promoter is also operably linked to the nucleic acid encoding the RecA protein and the operator.) In some embodiments, the operator binds a repressor. In some embodiments, the operator binds the lac repressor.

[0091] In some embodiments, the invention provides a *Bacillus anthracis* bacterium (and strain) that comprises a nucleic acid that produces a RecA antisense RNA upon transcription, wherein the nucleic acid is operably linked to an operator which binds a repressor or activator. (Typically a promoter is also operably linked to the nucleic acid encoding the RecA protein and the operator.) In some embodiments, the operator binds a repressor. In some embodiments, the operator binds the lac repressor.

[0092] The invention further provides a polynucleotide comprising a nucleic acid that produces a RecA antisense RNA upon transcription, wherein the nucleic acid is operably linked to an operator that binds a lac repressor. For instance, the invention provides a polynucleotide comprising a nucleic acid sequence complementary to a recA RNA transcript, or a portion thereof, which will hybridize thereto under cellular conditions, wherein the nucleic acid sequence is operably linked to an operator that binds a lac repressor (or is operably linked to both the operator and a promoter suitable for transcription of the nucleic acid sequence in the bacteria). An expression cassette, vector, and host cell comprising this polynucleotide is also provided.

[0093] In some embodiments, the modified *B. anthracis* bacterium or strain comprises an expression cassette that expresses a RecA antisense RNA.

[0094] In some embodiments, the inducible *recA* mutant comprises a *recA* gene which is operably linked to an inducible promoter or a repressible promoter. For instance, in some embodiments, the *recA* gene is operably linked to a regulatory sequence (such as a lac operator) which binds a transcriptional repressor (e.g., the lac repressor). In some embodiments, addition of an inducer molecule such as IPTG induces transcription of *recA*.

[0095] In some embodiments, the modification made to the *B. anthracis* strain or bacterium comprises a nucleic acid molecule encoding a RecA protein, wherein expression of the RecA protein is under the control of a transcriptional or translational activator or repressor (i.e., expression of the RecA protein is controlled (or modulated) by the activator or repressor). The control may be direct or indirect. In some embodiments, the expression is inducible. In some embodiments, the expression is repressible. In some embodiments, expression of the RecA protein is under the control of a transcriptional repressor (e.g., the lac repressor). In some embodiments, the nucleic acid encoding the RecA protein is operably linked to an operator which binds the repressor or activator. For instance, the nucleic acid encoding the RecA protein may be operably linked both to a promoter and to an operator which binds a repressor or activator such as the lac repressor.

[0096] In some another embodiments, the present invention provides a *recA* gene under the control of a transcriptional or translational activator or repressor. The transcriptional activator can be activated during conditions where the *B. anthracis* is grown, and where the transcriptional activator can be deactivated at a later time, that is, when psoralen is introduced. Similarly, the transcriptional repressor can be engineered so that it is inactive when the *B. anthracis* is grown, and so that it is active when the *B. anthracis* is treated with psoralen. The *recA* gene of the present invention can be functionally (or operably) linked with a nucleic acid encoding anti-sense nucleic acid, in a way that allows expression of RecAduring growth of the *B. anthracis*, but inhibits or prevents expression of RecA during and after treatment with psoralen.

[0097] The present invention provides a transcriptional activator, an transcriptional repressor, an translational activator, and an translatable repressor, e.g., for regulating RecA

expression. The present invention also provides a nucleic acid encoding an antisense polynucleotide, where the antisense polynucleotide can inhibit translation of RecA.

[0098] Provided in some embodiments is a transcriptional or translational activator or repressor that supports expression of RecA during growth or preparation of a culture of *B. anthracis* cells, where the modulator (i.e., effector) of the repressor (or activator) is withdrawn (or terminated) shortly prior to administrating a DNA-modifying agent. Removal or termination of the modulator of the repressor (or activator), shortly before administering the DNA-modifying agent, serves to reduce RecA-mediated repair of the DNA, thus allowing DNA modification at relatively low concentrations of the DNA-modifying agent. In some embodiments, the repressor is a transcriptional repressor.

[0099] Also provided is a transcriptional or translational repressor (or activator), where the modulator of the repressor (or activator) can be added after growth or preparation of a culture of *B. anthracis* cells. In some embodiments, the modulator is introduced shortly before administering a DNA-modifying agent. Here, the repressor (or activator) provokes a reduction in RecA expression in the *B. anthracis* cell, thus allowing DNA modification at relatively low concentrations of a DNA modifying agent. In some embodiments, a transcriptional repressor is utilized.

[0100] As indicated above, it is also possible to construct the *Bacillus anthracis* strain such that the *recA* is under the control of the lac repressor, such that expression of *recA* can be induced by isopropyl-beta-D-thiogalactopyranoside (IPTG) when desired, but not during photochemical inactivation or post-immunization. The possibility of *recA* expression can then be eliminated for the inactivation and/or immunization steps by withholding further IPTG from the strain and/or eliminating IPTG from the strain's environment.

[0101] For instance, to generate a *Bacillus anthracis* bacterium in which *recA* is repressed by the lac repressor for use in the vaccines of the invention, two expression cassettes are introduced into the genome of the bacterium (e.g., *Bacillus anthracis ΔuvrAB*). The first expression cassette enodes the lac I^q protein under the control of a highly-active constitutive promoter, for example, but not limited to the *B. anthracis* promoters P*amy*, P*ntr*, or the *pagA* promoter. The second cassette expresses RecA anti-sense RNA, also under the control of a selected promoter, for example, but not limited to, a *B. anthracis* promoter such as the P*amy*, P*ntr*, or *pagA* promoter, but, importantly, the *lacOip* operator is placed at the 3' end of the

selected promoter. Thus, expression of the RecA anti-sense RNA is prevented in the presence of the lac I^q protein. In this configuration, functional RecA protein is produced under normal growth conditions, due to constitutive expression of the lac I^q protein which prevents expression of the RecA anti-sense RNA. Addition of IPTG to the culture will result in binding of this inducer molecule to the lac I^q repressor, and prevent it from binding to the *lacOip* operator. This results in the high-level synthesis of RecA anti-sense RNA and, in turn, inhibition of translation of RecA protein, mediated through complementary binding to RecA message. The expression cassettes can be assembled onto the pKSV7 integration vector and introduced into *Bacillus anthracis* Δ*uvrAB* at any desired location, but desirably within intergenic regions of the bacterial chromosome. In this setting, the expression of *recA* is shut-off by addition of IPTG to the fermentation culture prior to illumination with UVA light.

[0102] In some embodiments, the *B. anthracis* strain is an inducible *recA* mutant which is under control of the lac repressor. In some embodiments, the *recA* promoter of *Bacillus anthracis* is engineered to contain a downstream *lacOip* site. An expression cassette is introduced into *Bacillus anthracis* encoding *lacI*^{q} under the control of a highly expressed constitutive promoter, for example, but not limited to the promoters for the *B. anthracis* genes *agA*, *ntr*, or *amy*. In this configuration, functional RecA protein is produced only in the presence of IPTG or other inducer molecule. Upon removing the inducer, *recA* transcription is blocked and the amount of RecA protein will decrease depending on its half-life in the bacterium. In this configuration, IPTG is present when it is desirable to culture the cells normally, and it is removed when it is desired to introduce DNA modifications and abrogate DNA repair. The expression cassette encoding *lacOip* can be assembled onto the pKSV7 integration vector and introduced into *Bacillus anthracis* at any desired location, but desirably within intergenic regions of the bacterial chromosome.

[0103] The present invention provides an engineered *E. coli* lac repressor and operator, for use in *B. anthracis*. See, e.g., Yansura and Henner (1984) Proc. Natl. Acad. Sci. USA 81:439-443, disclosing the *E. coli* lac repressor and operator in *B. subtilis*. However, a variety of other regulatory elements useful as part of inducible and/or repressible expression systems in bacteria are known in the art. For instance, heat-inducible expression systems for use in *B. anthracis* are available (Baillie, et al. (1998) FEMS Microbiol. Lett. 163:43-47). A number of response elements are disclosed for *B. subtilis*. These elements respond to, e.g., heat shock,

cold-induction, salt stress, ethanol, oxygen starvation, reactive oxygen (e.g., peroxide), nutrient starvation, nutrient addition (e.g., aminobutyrate, glucose, maltose, or arabinose), nitrogen limitation, alkali shift (sudden shift to pH 8.9). For example, a cold response element in *B. subtilis* is induced with a shift down in temperature from 37 degrees to 18 degrees (see, e.g., Hecker, et al. (1996) Mol. Microbiol. 19:417-428; Yasbin, et al. (1992) Mol. Microbiol. 6:1263-1270; Mongkolsuk and Helmann (2002) Mol. Microbiol. 45:9-15; Sakamoto and Murata (2002) Curr. Opin. Microbiol. 5:208-210; Fisher (1999) Mol. Microbiol. 32:223-232; Nickel, et al. (2004) Mol. Genet. Genomics 272:98-107; Chan and Lim (2003) J. Mol. Biol. 333:21-31; Atalla and Schumann (2003) J. Bacteriol. 185:5019-5022; Kawai, et al. (2003) Mol. Microbiol. 47:1113-1122; Kaan, et al. (2002) Microbiol. 148:3441-3455; Belitsky and Sonenshein (2002) Mol. Microbiol. 45:569-583; Fuangthong, et al. (2002) J. Bacteriol. 184:3276-3286; Stulke, et al. (1997) Mol. Microbiol. 25:65-78; Mota, et al. (2001) J. Bacteriol. 183:4190-4201; Noone, et al. (2000) J. Bacteriol. 182:1592-1599; Schonert, et al. (1999) Res. Microbiol. 150:167-177).

[0104] In some embodiments, the repair deficient mutant lacks the ability to make a photolyase which repairs pyrimidine dimers. For example, the mutation may be in a gene equivalent to a *phrB* gene. Such a mutant could be used in conjuction with ultraviolet irradiation (e.g. UVB, UVC) of the bacteria to produce pyrimidine dimers in the bacterial nucleic acid.

[0105] In one embodiment, the bacteria comprises at least one mutation that reduces (preferably, significantly reduces) the ability of the bacteria to repair modifications to their nucleic acid in combination with at least one mutation not related to repair mechanisms.

[0106] In some embodiments, the invention provides a *Bacillus anthracis* strain which is defective with respect to at least one DNA repair enzyme (relative to wild type). In some embodiments, the strain that is defective with respect to at least one DNA repair enzyme is attenuated for DNA repair relative to wild type. In some embodiments, the capacity of the bacteria for DNA repair by at least one DNA repair pathway is reduced by at least about 10%, at least about 25%, at least about 50%, at least about 75%, or at least about 90% relative to wild type. In some embodiments, the capacity of the bacteria for DNA repair by at least one DNA repair pathway is reduced by at least about 10% relative to wild type. In some embodiments, the capacity of the bacteria for DNA repair by at least one DNA repair pathway is reduced by at least about 25% relative to wild type. In some embodiments, the capacity of the bacteria for DNA repair by at least one DNA repair pathway is reduced by at least about 50% relative to wild type.

In some embodiments, the capacity of the bacteria for DNA repair by at least one DNA repair pathway is reduced by at least about 75% relative to wild type. Methods for assessing the ability of a strain to effect DNA repair are well known to those of ordinary skill in the art. In some embodiments, the strain is defective with respect to one or more of the following enzymes: PhrB, UvrA, UvrB, UvrC, UvrD, and RecA. In some embodiments, the strain is defective with respect to UvrA, UvrB, or both enzymes. In some embodiments, the strain is defective with respect to UvrC. In some embodiments, the strain is defective with respect to RecA. In some embodiments, the bacteria comprise a genetic mutation in one or more gene selected from the group consisting of *phrB, uvrA, uvrB, uvrC, uvrD* and *recA*. In some embodiments, the bacteria comprise genetic mutations in both *uvrA* and *uvrB*. In some embodiments, the bacteria comprise a genetic mutation in *uvrC*. In some embodiments, the *Bacillus anthracis* is a *uvrAB* deletion mutant. In some embodiments, the bacteria comprise a genetic mutation in *recA*.

[0107] Thus, the invention provides an isolated *Bacillus anthracis* strain, comprising a genetic mutation that attenuates its ability to repair its nucleic acid (i.e., is attenuated for DNA repair). In some embodiments, the mutant strain is defective with respect to at least one DNA repair enzyme (such asUvrA and/or UvrB, or UvrC). In some embodiments, the mutant strain comprises a genetic mutation in the *uvrA* gene and/or the *uvrB* gene. In some embodiments, the *uvrA* gene, the *uvrB* gene , or both genes are deleted. (The coding sequence of the *uvrA* gene is shown as SEQ ID NO:1, below, and the coding sequence of the *uvrB* gene is shown as SEQ ID NO:2, below.) In some embodiments, the modified *Bacillus anthracis* strain is the *Bacillus anthracis Sterne ΔuvrAB* strain deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia, 20110-2209, United States of America, on February 20, 2004, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and identified by accession number PTA-5825, or a mutant of the deposited strain which is defective with respect to UvrA and UvrB. In some embodiments, the modified strain is the *Bacillus anthracis Sterne ΔuvrAB* strain deposited with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia, 20110-2209, United States of America, on February 20, 2004, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure, and identified by accession number PTA-5825. In some embodiments, the mutant strain comprises a genetic mutation in the *uvr*C gene.

[0108] In some embodiments, the mutant strain is attenuated with respect to RecA. In some embodiments, the mutant strain comprises a genetic mutation in the *recA* gene. In some embodiments the mutant strain comprises a conditional *recA* gene. In some embodiments, the mutant strain comprises a mutation in the *recA* gene that makes expression of the *recA* protein temperature sensitive. In some alternative embodiments, a mutant strain of *B. anthracis* is constructed which comprises a repressible *recA* gene (e.g, the *recA* gene is under control of the lac repressor (inducible by IPTG), permitting expression of *recA* during growth, but not during inactivation (such as with S-59/UVA) and/or post-immunization). In some embodiments, the mutant strain comprises one or more mutations in the *lef* gene, *cya* gene, or both genes, that decreases the toxicity of the strain relative to the non-mutated strain. In some embodiments, the modified strain is also asporogenic.

[0109] The invention further provides an isolated *Bacillus anthracis* strain comprising a temperature sensitive *recA* gene. In some embodiments, the strain is defective with respect to UvrA and/or UvrB. In some embodiments, the strain is defective with respect to UvrC. In some embodiments, the strain is asporogenic. In some embodiments, the strain comprises a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased.

[0110] Methods of altering the genome of *Bacillus anthracis* are known to those skilled in the art. One method of generating mutations in *Bacillus anthracis* is by allelic exchange using an allelic exchange vector known to those in the art. An exemplary allelic exchange plasmid is pKSV7 described in Camilli et al., Molecular Microbiology, 8:143-147 (1993). As a first step in generating a mutant *Bacillus anthracis*, the region of the genome to be deleted or otherwise mutated and approximately 1000 bps both upstream and downstream of the *B. anthracis* genome is PCR-amplified and then cloned into the pKSV7 plasmid vector (or an analogous vector). (A *Bacillus* genera-specific or *B. anthracis*-specific temperature (ts) replicon may be substituted for the *Listeria* ts replicon present in the pKSV7 allelic exchange plasmid vector.) Restriction endonuclease recognition sites in the region to be deleted or mutated may be used to delete the desired portion of the targeted gene in the region. Alternatively, a portion of the targeted gene

within the region may be removed and replaced with sequences containing the desired mutation or other alteration. The region of the *B. anthracis* genome that is amplified can be altered, for instance, using restriction enzymes or a combination of restriction enzymes and synthetic gene sequences, before or after cloning into the allelic exchange plasmid. In some embodiments, the sequence may be altered as a PCR amplicon and then cloned into pKSV7. In alternative embodiments, the amplicon is first inserted into another plasmid first and then altered, excised, and inserted into pKSV7. Alternatively, the PCR amplicon is inserted directly into the pKSV7 plasmid and then altered, for instance, using convenient restriction enzymes. The pKSV7 plasmid containing the altered sequence is then introduced into *B. anthracis*. This can be done via electroporation. The bacteria are then selected on media at a permissive temperature in the presence of chloramphenicol. This is followed by selection for single cross-over integration into the bacterial chromosome by passaging for multiple generations at a non-permissive temperature in the presence of chloramphenicol. Lastly, colonies are passaged for multiple generations at the permissive temperature in media not containing the antibiotic. Some additional details regarding this method as applied to the deletion of *uvrAB* from *Bacillus anthracis* are provided, by way of non-limiting example, in Example 1 and Example 2, below. Example 4, below, also provides an example of the use of pKSV7 to delete *spoIIE*.

[0111] One of ordinary skill in the art will be able to readily adapt the pKSV7 allelic exchange method (or common methods known in the art) to introduce any of the mutations described herein into *Bacillus anthracis*, including, but not limited, to the *recA* mutations described herein.

[0112] Confirmation of the presence of a desired mutation in a bacterium can be determined by PCR and/or sequencing using standard methods known in the art. For the PCR or sequencing, primers are used which are complementary to those regions flanking the area of interest on the chromosome where the deletion or other mutation was intended. Alternatively, the presence of the desired mutation can be determined by functional assay. For instance, the presence of nucleotide excision repair mutation such as deletion of *uvrAB* can be assessed using an assay which tests the ability of the bacteria to repair its nucleic acid using the nucleotide excision repair (NER) machinery and comparing that ability against wild-type *Bacillus anthracis*. Such functional assays are known in the art. For instance, cyclobutane dimer excision or the excision of UV-induced (6-4) products can be measured to determine a deficiency

in an NER enzyme in the mutant (see, e.g., Franklin et al., *Proc. Natl. Acad. Sci. USA*, 81: 3821-3824 (1984)). Alternatively, survival measurements can be made to assess a deficiency in nucleic acid repair. For instance, the bacteria can be subjected to psoralen/UVA treatment and then assessed for its ability to proliferate and/or survive in comparison to wild-type.

[0113] Methods for assessing activity and expression, as well as for measuring the stimulation, inhibition, or inactivation, of nucleic acid repair proteins and nucleic acid repair pathways are known in the art (see, e.g., Lin and Sancar (1990) J. Biol. Chem. 265:21337-21341; Smith, et al. (2002) J. Bacteriol. 184:488-493; Ramaswamy and Yeung (1994) J. Biol. Chem. 269:485-492; Crowley and Hanawalt (2001) Mutat. Res. 485:319-329; Bierne, et al. (1997) Mol. Microbiol. 26:557-567; Courcelle and Hanawalt (2001) Proc. Natl. Acad. Sci. USA 98:8196-8202). Expression and/or activity can be assessed by standard assays, e.g., Northern blots, antibody-based assays, and *in vitro* or *in vitro* functional assays using UV-light or chemically damaged plasmid substrates (see, e.g., Mu and Sancar (1997) Prog. Nucleic Acid Res. Mol. Biol. 56:63-81; Sancar (1996) Annu. Rev. Biochem. 65:43-81; Clark (1991) Biochemie 73:523-532).

[0114] Additional information regarding the mutation of microbes such as *Bacillus anthracis* to render them defective with respect to nucleic acid repair is provided in U.S. Provisional Application No. 60/446,051, filed February 6, 2003; U.S. Provisional Application No. 60/449,153, filed February 21, 2003; U.S. Provisional Application No. 60/490,089, filed July 24, 2003; U.S. Provisional Application No. 60/511,869, filed October 15, 2003; U.S. Patent Application Serial No. 10/773,618, filed February 6, 2004 (U.S. Patent Publication No. 2004/0197343 A1); and the U.S. Patent Application Serial No., 10/883,599, filed June 30, 2004, the disclosures of each of which are incorporated by reference herein in their entirety.

[0115] In some preferred embodiments, the nucleic acid of the strain that is defective with respect to nucleic acid repair has been modified so that the strain is also attenuated for proliferation. Methods of modifying the nucleic acid of a strain so that the strain is attenuate for proliferation are described herein.

[0116] Either as an alternative to or in addition to one or more of the nucleic acid repair mutations described above (e.g., *recA*, *uvrA*, *uvrB*, *uvrC*, and *uvrAB*), in some embodiments the modified *B. anthracis* strains comprise other mutations.

[0117] In some embodiments, the *B. anthracis* bacteria are defective with respect to the formation of spores (i.e., are sporulation-deficient). For instance, in some embodiments, the *B.*

anthracis strains are asporogenic. In some embodiments, the bacteria are defective with respect to a sporulation protein. In some embodiments, the *B. anthracis* bacteria comprise an inhibiting mutation in, or a modification that attenuates expression of, at least one sporulation gene, a gene required or used for sporulation, e.g., *spoIIE*, *spoOA*, *spoIVB*, *spoIIA*, and *spoIIG* (see, e.g., Liu, et al. (2003) Nucleic Acids Res. 31:6891-6903; Guidi-Rontani, et al. (1999) Mol. Microbiol. 33:407-414; Brown, et al. (1994) Mol. Microbiol. 14:411-426; Worsham et al. (1999) Can. J. Microbiol., 45:1-8). In some embodiments, the *B. anthracis* strain comprises a mutation in *spoIIE*. In some embodiments, the asporogenic strains are defective with respect to SpoIIE, a protein phosphatase that causes release of a sigma factor called sigma F (Hilbert, et al., *J. Bacteriol.*, 185: 1590-8 (2003)). In some embodiments, the strains are rendered asporogenic by mutation of the *spoIIE* gene. In some embodiments, part or all of the *spoIIE* gene is deleted. In some embodiments, the *spoIIE* gene is deleted. In some embodiments, the apsorogenic or sporulation-deficient bacteria and/or strain does not comprise a mutation in either *spoOA* or *spoIVB*. In some embodiments the bacteria and/or strain are not defective with respect to SpoOA or SpoIVB.

[0118] In some embodiments, expression and/or activity of a sporulation gene or production of a *B. anthracis* spore or spore component is reduced in the sporulation-deficient mutant by at least about 25%, at least about 50%, at least about 75%, at least about 90%, at least about 97%, or at least about 100%.

[0119] Expression and/or activity of a sporulation gene or production of a *B. anthracis* spore or spore component, can be assessed by, methods known in the art. For instance, expression or activity can be assessed, by e.g., a Congo red agar assay; PCR assays; a spore germination assay; Anthrax Bio-Threat Alert® test strips (Tetracore, Gaithersberg, MD); antibody-based assays; DNA binding assays; dipicolinic acid assays; and mouse assays (see, e.g., Worsham and Sowers (1999) Can. J. Microbiol. 45:1-8; Drago, et al. (2002) J. Clin. Microbiol. 40:4399; King, et al. (2003) J. Clin. Microbiol. 41:3454-3455; Hartley and Baeumner (2003) Analyt. Bioanal. Chem. 376:319-327; Carl, et al. (1992) J. Infect. Dis. 165:1145-1148; Zhou, et al. (2002) Proc. Natl. Acad. Sci. USA 99:5241-5246; Bruno and Kiel (1999) Biosens. Bioelectron. 14:457-464; Beverly, et al. (1996) Rapid Commun. Mass Spectrom. 10:455-458; Book, et al. (2001) J. Med. Microbiol. 50:702-711).

[0120] Accordingly, in some embodiments, the invention provides an isolated, asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair. In one embodiment, the strain is defective with respect to SpoIIE. In some embodiments, the strain is also defective with respect to UvrA and/or UvrB (e.g., comprises a mutation in the *uvrAB* gene). In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC* gene). In some embodiments the strain is also defective with respect to RecA. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the strain comprises one or more mutations in the *lef* gene and/or the *cya* gene so that the toxicity of the strain is decreased. A composition, such as a vaccine composition, comprising a bacterium of the strain is also provided. Methods of inducing an immune response in a host comprising administering to the host an effective amount of the composition are also provided. In addition, methods of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of the strain are also provided.

[0121] In some embodiments, the *Bacillus anthracis* bacteria comprise are defective with respect to one or more toxins, such as lethal factor (LF) or edema factor (EF). In some embodiments, the bacteria comprise a mutation in the *lef* gene, or the *cya* gene, or both. In some embodiments, the mutation in the *lef* gene or the *cya* gene (or both) reduces the toxicity of the strain. In some embodiments, the toxicity of the strain is reduced by at least about 5%, at least about 10%, at least about 20%, at least about 30%, at least about 50%, or at least about 75%. The present invention also encompasses, without limitation, embodiments of a *B. anthracis* strain comprising an inhibiting mutation in, or a modification that attenuates expression of, at least one toxin gene, such as the *lef* gene, the *cya* gene, or both the *lef* gene and the *cya* gene. A "toxin gene" encompasses, but is not limited to, a gene that mediates, e.g., edema, shock, damage to a host cell, pathogenesis, or death to a host cell or organism (see, e.g., Brossier, et al. (2000) Infection Immunity 68:1781-1876). *B. anthracis* toxin genes include, e.g., the *lef* gene and *cya* gene. In some embodiments, the mutation is a point mutation. In other embodiments, the mutation is a deletion.

[0122] Toxicity of a modified strain of *B. anthracis* relative to wild-type and/or relative to the strain without the modification can be assessed using methods known in the art, such as determination of LD_{50} in an animal model. See, e.g., Section I.B, below.

[0123] The present invention encompasses, without limitation, embodiments of a *B. anthracis* strain comprising an inhibiting mutation in, or a modification that attenuates expression of, at least one sporulation gene or toxin gene, such as the *spoIIE* gene; the *lef* gene; the *cya* gene; in both the *spoIIE* gene and the *lef* gene; in both the *spoIIE* gene and the *cya* gene; in both the *lef* gene and the *cya* gene; or in all three of the *spoIIE* gene, *lef* gene, and *cya* gene.

[0124] In some embodiments where the bacteria comprise a mutation in, or a modification that attenuates expression of, a toxin gene, the bacteria further comprise a nucleic acid encoding an antigen, wherein the nucleic acid is operably linked to a heterologous promoter. In some embodiments, the antigen is, or is derived from, a polypeptide encoded by, e.g., *pagA* gene; *lef* gene; *cya* gene; *pagA* gene and *lef* gene; *pagA* gene and *cya* gene; *lef* gene and *cya* gene; or all three of *pagA* gene, *lef* gene, and *cya* gene. Where the heterologous promoter is operably linked to a nucleic acid encoding an antigen, the heterologous promoter is, in some embodiments, an inducible heterologous promoter.

[0125] In some embodiments, the modified *Bacillus anthracis* bacteria comprise at least one heterologous nucleic acid sequence (i.e., heterologous polynucleotide). In some embodiments, the bacteria comprise more than one heterologous nucleic acid sequence. The heterologous nucleic acid sequences are, in some embodiments, expression cassettes or expression vectors. Optionally, the heterologous nucleic acid sequences have been integrated into the *B. anthracis* chromosome.

[0126] Expression cassettes suitable for use in the *B. anthracis* are known to those of ordinary skill in the art. For instance, it is known that an expression cassette suitable for use in the bacteria typically comprises a polynucleotide encoding a polypeptide (e.g., a heterologous protein) and a promoter operably linked to the protein-encoding polynucleotide. The expression cassette optionally further comprises a polynucleotide encoding a signal peptide sequence, so that the expression cassette comprises a promoter, polynucleotide encoding a signal peptide sequence, and a coding sequence, all operably linked, so that the expression cassette encodes a fusion protein comprising both the signal peptide sequence and the desired polypeptide sequence. In addition, an expression cassette optionally comprises the following elements: (1) prokaryotic promoter; (2) Shine-Dalgarno sequence; (3) a polynucleotide encoding a signal peptide; and, (4) a polynucleotide encoding a polypeptide (such as a heterologous protein).

[0127] In some embodiments, the expression cassette may also contain a transcription termination sequence inserted downstream from the C-terminus of the translational stop codon related to the heterologous polypeptide. For instance, a transcription termination sequence may be used in constructs designed for stable integration within the bacterial chromosome. While not required, inclusion of a transcription termination sequence as the final ordered element in a heterologous gene expression cassette may prevent polar effects on the regulation of expression of adjacent genes, due to read-through transcription. Appropriate sequence elements known to those who are skilled in the art that promote either rho-dependent or rho-independent transcription termination can be placed in the heterologous protein expression cassette.

[0128] In some embodiments, the promoter used in an expression cassette described herein is a constitutive promoter. In other embodiments, the promoter used in an expression cassette described herein is an inducible promoter. The inducible promoter can be induced by a molecule (e.g., a protein) endogenous to the bacteria in which the expression cassette is to be used. Alternatively, the inducible promoter can be induced by a molecule (e.g. a small molecule or protein) heterologous to the bacteria in which the expression cassette is to be used. A variety of inducible promoters are well-known to those of ordinary skill in the art.

[0129] Optionally, at the 3'-end of the promoter is a poly-purine Shine-Dalgarno sequence, the element required for engagement of the 30S ribosomal subunit (via 16S rRNA) to the heterologous gene RNA transcript and initiation of translation. The Shine-Dalgarno sequence has typically the following consensus sequence: 5'-NAGGAGGU-N_{5-10}-AUG (start codon)-3' (SEQ ID NO:3). There are variations of the poly-purine Shine-Dalgarno sequence.

[0130] Suitable signal sequences are also known to those of ordinary skill in art. For instance, a sequence encoding a secA1 signal peptide can be used in the expression cassette and/or expression vector. One example of a secA1 signal peptide suitable for use in *Bacillus anthracis* comes from the Pag (Protective Antigen) gene from *Bacillus anthracis* (MKKRKVLIPLMALSTILVSSTGNLEVIQAEV (SEQ ID NO:4); signal peptidase cleavage site represented by ': IQA'EV (SEQ ID NO:5)).

[0131] The expression cassette is optionally contained within an expression vector, such as, but not limited to, a plasmid. In some embodiments, the vector is an integration vector. (In some embodiments, the strain comprises an integrated expression cassette.)

[0132] For instance, expression vectors suitable for use in *B. anthracis* are known to those of ordinary skill in the art. There are a variety of vectors suitable for use as a plasmid construct backbone for assembly of the expression cassettes. A particular plasmid construct backbone is selected based on whether expression of the heterologous gene from the bacterial chromosome or from an extra-chromosomal episome is desired.

[0133] Incorporation of the heterologous gene expression cassette into the bacterial chromosome of *B. anthracis* can be accomplished with an integration vector that contains an expression cassette for a phage integrase that catalyzes sequence-specific integration of the vector into the *B. anthracis* chromosome. The integrase and attachment site of a *B. anthracis* phage can be used to derive an integration vector to incorporate desired antigen expression cassettes into the vaccine composition. For instance, the integrase and attachment site from the *B. anthracis* temperate phage w-alpha can be used to derive a *B. anthracis* specific integration vector (see, e.g., McCloy, E.W. 1951. Studies on a lysogenic Bacillus strain. I. A bacteriophage specific for *Bacillus anthracis*. J. Hyg. 49:114-125, incorporated by reference herein in its entirety).

[0134] Alternatively, incorporation of an antigen expression cassette into the *B. anthracis* chromosome can be accomplished through alleleic exchange methods, known to those skilled in the art. In particular, compositions in which it is desired to not incorporate a gene encoding an antibiotic resistance protein as part of the construct containing the heterologous gene expression cassette, methods of allelic exchange are desirable. For example, the pKSV7 vector (Camilli et. al. *Mol. Microbiol.* 1993 8,143-157), contains a temperature-sensitive *Listeria*-derived Gram positive replication origin which is exploited to select for recombinant clones at the non-permissive temperature that represent the pKSV7 plasmid recombined into the *Listeria* chromosome. The pKSV7 allelic exchange plasmid vector contains a multiple cloning site sequence in order to facilitate construction of plasmids containing the heterologous protein expression cassette, and also a chloramphenicol resistance gene. For insertion into the *Bacillus anthracis* chromosome, the heterologous antigen expression cassette construct may be flanked by approximately 1 kb of chromosomal DNA sequence that corresponds to the precise location of desired integration. The pKSV7-heterologous protein expression cassette plasmid may be introduced into a desired bacterial strain by electroporation, according to standard methods for electroporation of Gram positive bacteria. A non-limiting example of a method of effecting

allelic exchange in *B. anthracis* using the pKSV7 vector is provided in Example 2 below. This result demonstrates that the pKSV7 vector-based technique of allelic exchange can be used to effect genetic modification in *Bacillus* species, which, like *Listeria*, are low G+C content organisms. In particular, allelic exchange using the pKSV7 vector can be used in strains of *B. anthracis* to delete or modify DNA repair genes, such as UvrAB, or to add a desired antigen expression cassette at any desired location within the bacterial chromosome.

[0135] An isolated *Bacillus anthracis* strain comprising a heterologous expression cassette comprising a nucleic acid sequence encoding protective antigen (*pagA*) is provided, wherein the nucleic acid sequence encoding the protective antigen is operably linked to an inducible promoter. In some embodiments, the nucleic acid sequence encoding protective antigen is operably linked to an SOS regulatory sequence, such as an SOS box. In some embodiments, the expression of the protective antigen is induced by treatment of the strain with a nucleic acid targeting compound that modifies the nucleic acid of the strain (see below for further information regarding nucleic acid targeting compounds). For instance, in some embodiments, expression is induced by treatment with a psoralen, such as S-59, and ultraviolet (UVA) light. In some embodiments, the expression cassette is on a plasmid or vector. In some embodiments, the expression cassette is integrated in the genomic DNA. In some embodiments, the expression cassette is produced in the genomic DNA (for instance by insertion of appropriate regulatory sequences so that they are operably linked to *pagA*).

[0136] In some embodiments, the protein that is expressed by the nucleic acid sequence on the heterologous expression cassette is a fragment or variant of protective antigen. In some embodiments, the functional fragment or variant is an immunogenic fragment or variant of protective antigen.

[0137] In some embodiments, the nucleic acid sequence encoding protective antigen may be operably linked to an SOS regulatory sequence. In some embodiments, expression of protective antigen is under control of an SOS regulatory sequence. In some embodiments, the strain comprises a mutation that attenuates the ability of the strain to repair its nucleic acid. For instance, in some embodiments, the strain is defective with respect to at least one DNA repair enzyme, such as UvrA, UvrB, or both UvrA and UvrB. In some embodiments, the strain comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene. In some embodiments, the strain is defective with respect to UvrC (e.g., comprises a mutation in the *uvrC*

gene). In some embodiments, the strain is defective with respect to RecA. A strain that is defective with respect to RecA can, by way of example, comprise a mutation in the *recA* gene, comprise a *recA* gene under control of a repressible promoter, or comprise a temperature sensitive *recA* gene. In some embodiments, the strain comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain. In some embodiments, the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation. In addition, the strain is, in some embodiments, asporogenic (e.g., a *spoIIE* mutant).

[0138] Accordingly, the invention also provides an isolated *Bacillus anthracis* strain comprising a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence. In some embodiments, the heterologous expression cassette is integrated into the *B. anthracis* chromosome. A Lex A repressor site exists upstream of the promoter for the *B. anthracis recA* and *uvrAB* genes, which are part of the SOS regulon. The regulatory sequences from these *B. anthracis* genes or from the corresponding genes in related bacteria such as *B. subtilis* can be placed on the heterologous expression cassette upstream of the protective antigen coding sequence and a suitable promoter, including, as non-limiting examples, *B. anthracis* promoters such as P*ntr*, P*amy*, or the PagA promoter. A description of the construction of an exemplary *Bacillus anthracis* strain that expresses protective antigen under control of the SOS regulatory sequence is provided in the specific example, Example 7, below.

[0139] LexA binds to a specific DNA sequence (the "SOS box") upstream of the genes it regulates, and represses transcription. RecA is one of the genes regulated in this manner. Exposure to DNA damage, e.g., UV light and/or psoralen, activates RecA coprotease activity which then triggers the cleavage of LexA. Once LexA is cleaved, LexA is released from its binding sites, where this release results in increased expression of the regulated genes. These regulated genes include *recA*, as mentioned above, as well as LexA, and 20-40 other genes. In other words, the SOS genes are regulated by two proteins, LexA and RecA, which themselves are products of SOS-regulated genes. A number of SOS boxes have been identified, where these SOS boxes comprise imperfect pallindromic sequences (see, e.g., Davis, et al. (2002) J. Bacteriol. 184:3287-3295; Movahedzadeh, et al. (1997) Microbiology 143:929-936; Winterling, et al. (1997) J. Bacteriol. 179:1698-1703; Winterling, et al. (1998) J. Bacteriol. 180:2201-2211; Cheo, et al. (1993) J. Bacteriol. 175:5907-5915; Campoy, et al. (2002) Microbiology 148:3583-

3597; Lovett, et al. (1993) J. Bacteriol. 175:6842-6849; Lovett, et al. (1994) 176:4914-4923; Microbiology 143:885-890; Miller, et al. (1996) J. Biol. Chem. 271:33502-33508). The reagents and methods of the present invention encompass regulatory sequences, e.g., imperfect palindromes, associated with or operably linked with various SOS response genes of *B. anthracis*, including the *B. anthracis recA* gene. Provided are methods of engineering an SOS box sequence, such as the following imperfect palindrome (a putative SOS box), or an imperfect palindrome associated with other *B. anthracis* SOS genes, to be operably linked with the *recA* gene, and/or with a nucleic acid such as the *pagA* gene, that encodes an antigen. The present invention further contemplates SOS response elements that are perfect palindromes, that overlap translation start sites, and that do not contain a palindrome. The present invention further contemplates the use of SOS response elements from other bacteria, especially other *Bacillus* bacteria, for use in the modified *Bacillus anthracis* bacteria of the present invention.

[0140] The present invention comprises nucleic acids, and methods, that utilize an imperfect palindrome that occurs at about 64 base pairs (bp) upstream of the start codon of recA coding sequence in *Bacillus anthracis* (GenBank Acc. No. NC_007530). This palindrome appears to be an SOS box. This palindrome (26 bp long) resides at nucleotide 3,591,817 to 3,591,843, of the *B. anthracis* genome (NC_007530). Functioning of this 26 bp imperfect palindrome, and variations of this palindromic sequence, is assessed by measuring expression of a nucleic acid encoding a reporter polypeptide, e.g., beta-galactosidase, where the 26 bp imperfect palindrome resides upstream (5-prime to) the nucleic acid encoding the reporter polypeptide. The regulatory function of the 26 bp imperfect palindrome, or of another *B. anthracis*-derived palindromic sequence, or of a *B. subtilis*-derived palindromic sequence, is determined using plasmids, where a *B. anthracis* strain harbors the plasmid. The ability of the putative regulatory sequence to increase expression of the reporter gene in response to an effector of DNA damage such as treatment with UV-light and/or psoralen that triggers the SOS response can be determined by measuring protein expression levels of the reporter polypeptide in the presence and absence of such an effector of DNA damage and comparing the levels of expression using methods standard in the art.

[0141] In some embodiments, UV-light and/or psoralen treatment, or another effector of DNA damage stimulates at least a 10% increase in expression of the reporter polypeptide, at least a 20% increase, at least a 30% increase, at least a 40% increase, at least a 50% increase, most at

least a 60% increase, at least a 70% increase, at least an 80% increase, or at least a 90% increase, where expression can be of a nucleic acid encoding, e.g., a reporter polypeptide, or an antigen derived from *B. anthracis* such as protective antigen (PA). In some embodiments, treatment stimulates at least about a 2-fold increase or treatment stimulates at least about a 10-fold increase. In some embodiments, treatment stimulates at least about a 20-fold increase, at least about a 50-fold increase, at least about a 100-fold increase, at least about a 200-fold increase, or at least about a 1000-fold increase, or more, where expression can be of a nucleic acid encoding, e.g., a reporter polypeptide, or an antigen derived from *B. anthracis* such as protective antigen (PA).

[0142] The present invention further provides an additional sequence appearing to be SOS box residing just upstream of the LexA coding region, at nucleotide 3,453,870 to 3,453,883 of GenBank Acc. No. NC_007530 (*Bacillus anthracis*). The sequence is: ATGTTTTTTCACAT (SEQ ID NO:46). The LexA coding region in *Bacillus anthracis* is nucleotides 3,453,933 to 3,454,553. The LexA gene contains an SOS response element (Dullaghan et al. (2002) Microbiology 148:3609-3615). The present invention also provides a consensus sequence ATGTNNNNNNACAT (SEQ ID NO:47) for use in *Bacillus anthracis*.

[0143] Also provided is another sequence which appears to be an SOS box in *Bacillus anthracis*, located just upstream of the ruvA gene. The palindromic region occurs at nucleotide 4,228,545 to 4,228,559, and has the structure: AAATGTTCTCCATTT (SEQ ID NO:48). The palindromic region occurs just upstream of the coding region of the *ruvA* gene. The *Bacillus anthracis* gene coding region is located at nucleotides complement to 4,227,852 to 4,228,469 to GenBank Acc. No. NC_007530. RuvA is an SOS response gene (Brooks et al. (2001) J. Bacteriol. 183:4459-4467). The present invention provides a consensus sequence: AAATGNNNNNCATTT (SEQ ID NO:49) for use in *Bacillus anthracis*.

[0144] The putative SOS boxes provided herein can be operably linked to promoter and a *recA* gene or an antigen coding sequence to place an antigen coding sequence, such as a nucleic acid encoding protective antigen, or another gene, such as a *recA* gene, under inducible control.

[0145] The present invention further contemplates identifying additional regulatory regions in the *B. anthracis* genome using, e.g., visual inspection and/or available algorithms. Use of algorithms has revealed regulatory motifs in the *B. subtilis* genome that are upstream of

known regulated *E. coli* genes, e.g., *LexA*, *Crp*, and *ArcA* in *B. subtilis* (see, e.g., McGuire, et al. (2000) Genome Res. 10:744-757).

[0146] In addition to providing regulatory sequences sensitive to DNA damage, the present invention provides regulatory sequences sensitive to, e.g., temperature, pH, osmotic changes, or alterations in the concentrations of oxygen, ions, or metabolites (see, e.g., Repoila and Gottesman (2003) J. Bacteriol. 185:6609-6614; Schofield, et al. (2003) Appl. Environ. Microbiol. 69:3385-3392; Hanna, et al. (2001) J. Bact. 183:5964-5973; Deuerling, et al. (1995) J. Bacteriol. 177:4105-4112). One or more of these regulatory sequences can be used to reduce or inhibit expression of a *B. anthracis* antigen or *B. anthracis* derived antigen during growth of the *B. anthracis*, but to stimulate or allow expression at a later time, e.g., during treatment with a DNA damaging agent or prior to administration to a human or animal subject.

[0147] A "heterologous promoter" which is operably linked to a specific gene or nucleic acid encompasses a promoter that is not normally operably linked in nature with that specific gene or nucleic acid, that is, a promoter that is not operably linked with that specific gene or nucleic acid in a parental bacterial strain or in a wild type bacterial strain. The heterologous promoter can be from the same bacterium, in other words, copied or duplicated from a promoter operably linked to a gene other than the above-identified specific gene or nucleic acid. In another embodiment, the heterologous promoter can be derived from a promoter from another species. In another embodiment, the heterologous promoter can be derived from a promoter from another strain of bacteria, so long as the promoter in the second strain is operably linked to a different gene than in the first strain, or the promoter in the second strain differs in sequence from the corresponding promoter in the first strain. The sequence of the heterologous promoter can be altered from that of a naturally occurring promoter, or it can be substantially or completely different from the sequence of any naturally occurring promoter.

[0148] The present invention further encompasses a *B. anthracis* strain comprising a nucleic acid encoding an antigen, wherein the nucleic acid is operably linked to a heterologous promoter. The antigen is, or is derived from, a polypeptide encoded by, e.g., *pagA* gene; *lef* gene; *cya* gene; *pagA* gene and *lef* gene; *pagA* gene and *cya* gene; *lef* gene and *cya* gene; or all three of *pagA* gene, *lef* gene, and *cya* gene. In some embodiments, the antigen is protective antigen. Where the heterologous promoter is operably linked a nucleic acid encoding an antigen, the heterologous promoter can be one that is an inducible heterologous promoter. In some

embodiments, the antigen encoded by the nucleic acid is full-length and/or the native sequence. In some other embodiments, the antigen is a variant and/or a fragment of the native antigen sequence. Software for determining, e.g., antigenic fragments, are available (see, e.g., Vector NTI® Suite (Informax, Inc., Bethesda, MD; Welling, et al. (1985) FEBS Lett. 188:215-218; Parker, et al. (1986) Biochemistry 25:5425-5432). In some embodiments the polynucleotide encoding the antigen comprises a mutation (e.g., a mutation which decreases the toxicity of the antigen).

[0149] In some embodiments, the inducible promoter is inducible by DNA damage. In some embodiments, the inducible promoter is inducible by one or more of the following: ultraviolet light; a nucleic acid cross-linking compound; ultraviolet light and a nucleic acid cross-linking compound; an SOS regulatory pathway; and/or a change or shift in temperature. In some embodiments, the inducible promoter (and the nucleic acid encoding the antigen) is operably linked to an SOS regulatory sequence, such as an SOS box.

[0150] In some embodiments, the modified strains of *B. anthracis* comprise heterologous expression cassettes which encode proteins such as cytolysins. Preferably, expression of the proteins enhances the potency of the immune response to the vaccine containing the bacteria upon administration to an animal. For instance, the modified bacteria of the invention optionally comprise heterologous nucleic acids such as expression cassettes or expression vectors that encode cytolysin. The modified bacteria of the invention optionally comprise heterologous nucleic acids, such as expression cassettes or expression vectors, which encode cytolysins. The heterologous cytolysin expressed by the *B. anthracis* strain in the vaccine is optionally Listeriolysin O (LLO), Streptolysin, or Perfringolysin, or a mutant version of Listeriolysin O (LLO), Streptolysin, or Perfringolysin.

[0151] For instance, the vaccine compositions of the present invention are optionally enhanced by the expression and secretion of Listeriolysin O (LLO), the cholesterol-dependent, pore-forming cytolysin from *Listeria monocytogenes*, by the *B. anthracis* strains within the vaccine. LLO is a critical virulence factor from *Listeria* because its expression in the phagolysosome allows Listeria to escape into the host cell cytosol. Importantly, it has been shown that expression of LLO by other microorganisms, such as *Bacillus subtilis* (Bielecki et al, Nature. 1990 345:175-6), *E. coli* (Higgins et al, Mol Microbiol. 1999 31:1631-1641), or *Mycobacterium bovis* BCG (Conradt et al, Microbes Infect. 1999 1:753-764), allows these

organisms or their protein antigens to enter the cytosol. This leads to improved antigen presentation via the MHC class I pathway and subsequent generation of CD8+ T cell responses.

[0152] In some embodiments, the LLO protein expressed by the bacteria is an LLO fusion protein that comprises a signal sequence, allowing it to be secreted from the intact bacteria. In this mode, the whole bacteria can gain access to the host cell cytosol. In some alternative embodiments, LLO protein that is expressed by the bacteria does not comprise a signal sequence, and the LLO protein is expressed and accumulated inside the bacteria without secretion. In this case, degradation and rupture of the bacteria within the phagolysosome ultimately leads to the release of proteins and/or antigens into the cytosol.

[0153] In some embodiments, the heterologous cytolysin that is expressed is a naturally occurring cytolysin. In other embodiments, the cytolysin that is expressed is a mutant form of the naturally occurring cytolysin. In some cases, the mutant cytolysin is more active than the naturally occurring cytolysin.

[0154] For instance, mutant forms of LLO that are more active than the native protein at neutral pH have been isolated from *Listeria* and characterized (Glomski et al., Infect Immun. 2003 71: 6754-6765). These mutant LLO proteins retain activity in the host cell cytosol and are thus cytotoxic to the host cell. The primary stimulus by which wild-type LLO activity is regulated is pH, which differs between the phagolysosome and the cytosol. Normally, LLO is active in the acidic environment of the phagolysosome, but is significantly less active in the cytosol. This enables *Listeria* to replicate and survive in the infected host cell long enough to infect adjacent cells by direct cell-to-cell spread. The increased activity of mutant LLO proteins in the neutral pH environment of the cytosol leads to premature host cell death and an enhanced immune response, including an anti-listerial response. In the context of the vaccine compositions described here, mutant cytolysins, such as the alternative LLO proteins described here, can be useful in non-Listerial species as a way of augmenting CD8+ T cell activation by promoting MHC class I antigen processing.

[0155] Thus, in some embodiments, the modified *B. anthracis* strains used in the vaccine compositions comprise a heterologous expression cassette which encodes a mutant LLO protein.

[0156] In some embodiments, at least one sequence in the expression cassette and/or vector contained within the modified *B. anthracis* is codon-optimized for expression in *B. anthracis*, as described, for instance, in the U.S. Patent Application Serial No. 10/883,599, filed

June 30, 2004, incorporated by reference herein in its entirety. In addition, codon optimization of sequences to be expressed in bacteria and suitable signal sequences are described in U.S. Serial No. 60/532,598, U.S. Serial No. 60/556,744, U.S. Serial No. 60/616,750, and U.S. Serial No. 11/021,441, each of which is incorporated by reference herein in its entirety.

B. Nucleic acid modification to attenuate proliferation

[0157] The invention further provides *B. anthracis* strains and/or bacteria, wherein the nucleic acid of the strains or bacteria are modified to attenuate the strains and/or bacteria for proliferation. For instance, in some embodiments, the modified *B. anthracis* strains of the invention which are attenuated for nucleic acid repair, are asporogenic, and/or express protective antigen under inducible control, are further modified to attenuate proliferation. In some embodiments, the modified *B. anthracis* strains have been modified by reaction with a nucleic acid targeting compound (also referred to herein as a "nucleic acid targeted compound") that reacts directly with the nucleic acid of the *B. anthracis* so that the bacteria are attenuated for proliferation.

[0158] Methods of attenuating the proliferation of microbes such as *Bacillus anthracis* by reaction with nucleic acid targeting compounds are described in U.S. Provisional Application No. 60/446,051, filed February 6, 2003; U.S. Provisional Application No. 60/449,153, filed February 21, 2003; U.S. Provisional Application No. 60/490,089, filed July 24, 2003; U.S. Provisional Application No. 60/511,869, filed October 15, 2003; U.S. Serial No. 10/773,618, filed February 6, 2004 (U.S. Patent Publication No. 2004/0 197343 A1); and in U.S. Patent Application Serial No. 10/883,599, filed June 30, 2004, the contents of each of which are incorporated by reference herein in their entirety.

[0159] In some embodiments, the nucleic acid of the *Bacillus anthracis* bacteria described herein has been modified so that the bacteria are attenuated for proliferation. In some embodiments, the nucleic acid of the bacterium has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid so that the bacterium is attenuated for proliferation. In some embodiments, the nucleic-acid targeted compound is a nucleic acid alkylator, such as beta-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In some embodiments, the nucleic acid targeted compound is activated by irradiation, such

as UVA irradiation. In some embodiments, the nucleic acid targeted compound is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen ("S-59").

[0160] In some embodiments, the nucleic acid of the bacteria and/or strain is modified so that the proliferation of the bacterium is attenuated. In some embodiments, the bacterial gene expression is substantially unaffected. In some embodiments, the bacterial gene expression is substantially unaffected so that an antigen is expressed at a level sufficient to stimulate an immune response to the bacteria upon administration of the bacteria to an individual. In some embodiments, the bacterial nucleic acid is modified by a method selected from the group consisting of exposing the bacteria to radiation and reacting the bacteria with a nucleic acid targeted compound that causes the modification of the bacterial nucleic acid. In a preferred embodiment, the bacterial nucleic acid is modified by reacting the bacteria with a nucleic acid targeted compound that reacts directly with the nucleic acid. In one embodiment, the nucleic acid targeted compound is targeted to the nucleic acid by a mode selected from the group consisting of intercalation, minor groove binding, major groove binding, electrostatic binding, and sequence-specific binding. In one embodiment, the nucleic acid targeted compound comprises a nucleic acid alkylator. In a preferred embodiment, the nucleic acid targeted compound is beta-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In one embodiment, the nucleic acid targeted compound that reacts directly with the nucleic acid reacts upon activation of the compound by irradiation, preferably by UVA irradiation. In one embodiment, the nucleic acid targeted compound activated by UVA irradiation is a psoralen. In a preferred embodiment, the psoralen is 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen. In one embodiment, the nucleic acid targeted compound indirectly causes the modification of the nucleic acid. In one embodiment, the nucleic acid targeted compound indirectly causes modification upon activation by irradiation, preferably by UVA irradiation.

[0161] In some embodiments, the modification of the nucleic acid in the bacterium and/or strain is direct. In other embodiments, the modification is indirect. In some embodiments, the modification is not a genetic modification. In some embodiments, the modification that attenuates the bacteria and/or strain for proliferation comprises breakage of strands of the DNA or cross-linking of the genomic DNA. In some embodiments, the modification constitutes DNA damage.

[0162] In some embodiments, the desired extent of modification is such that replication of the bacterium's genome is significantly attenuated while the production of proteins remains sufficiently active (i.e. the bacterium is metabolically active). It is to be understood that whatever the nature of the modification, the level of modification can be represented in terms of the number of modifications on average per base pair of the bacterial genome. For example, if the modification is due to covalent binding of a compound to the nucleic acid (adducts), the modification can be represented in terms of the average number of base pairs between adducts. In some embodiments, the bacteria of the invention can be modified to levels of about 1 modification per 10^3-10^8 base pairs, about 1 modification per 10^4-10^8 base pairs, about 1 modification per 10^4-10^7, about 1 modification per 10^5-10^7, or about 1 modification per 10^5-10^6 base pairs. In one embodiment, the level of modification is adjusted to the minimum amount required to block DNA replication in the bacterial population, such that the population shows no observable proliferation, while maintaining sufficient activity of transcription and translation of individual genes (i.e. maintains some metabolic activity) to achieve a safe and effective vaccine.

[0163] In some embodiments, the genomic DNA of the modified bacteria comprise at least one covalently linked nucleic acid targeting or cross-linking compound. In some embodiments, the genomic DNA comprises at most ten, often comprises at most about 20, more often comprises at most about 100, most often comprises at most about 200, generally comprises at most about 500, more generally comprises at most about 1000, most generally comprises at most about 2000, normally comprises at most about 5000, more normally comprises at most about 10,000, most normally comprises at most about 20,000, typically comprises at most about 50,000, more typically comprises at most about 100,000, most typically contains at most about 500,000, customarily contains at most about 1,000,000, more customarily contains at most about 2,000,000, or most customarily contains at most about 5,000,000, covalently linked nucleic acid targeting or cross-linking compounds. In one embodiment, the covalently linked nucleic acid targeting or cross-linking compound prevents or inhibits *B. anthracis* proliferation. In some embodiments, the present invention provides a *B. anthracis* wherein the genomic DNA is linked to at least one, often at least about ten, more often at least about 20, most often at least about 100, usually at least about 200, more usually at least about 1000, most usually at least about 2000, and conventionally at least about 10,000 covalently linked nucleic acid targeting or cross-linking compounds.

1. Attenuation of *B. anthracis* proliferation

[0164] In some embodiments, the present invention also involves the modification of bacterial nucleic acid in order to attenuate replication (i.e., proliferation) of the bacteria. (The terms replication and proliferation are used interchangeably herein.) This attenuation in replication can be used to increase the level of safety upon administration of the bacteria to individuals. The ability of a bacterium to proliferate can be measured by culturing a population of bacteria under conditions that provide normal growth. The normal growth of a population of bacteria is considered to be the growth of bacteria having no modifications to the nucleic acid of the bacteria. The modification of the bacterial genome will result in some attenuation so that the bacteria will not undergo normal growth. Some bacteria will form colonies that can be counted on solidified growth medium. Attenuation of the replication of the bacteria can thus be measured as a reduction in the number of colony forming units (CFU). A stock solution of the bacterial colony is serially diluted until the number of colony forming units can be easily measured (e.g. 50-500 CFU, on a 100 mM agar Media containing plate). Typically, dilutions are 10-fold and the number of colonies counted for one or more of the diluted samples is used to estimate the log titer of the sample. For example, an aliquot of diluted bacterial stock is plated on growth media and the resulting colonies are counted. The colony forming units per mL (CFU/mL) of the dilution is calculated, and the colony forming units per mL of the original stock (known as the titer) is calculated from the dilution. The log number is known as the log titer. As an example, 24 colony forming units on plating a 0.2 mL aliquot of a 1 $x10^5$ dilution gives a 1.2 x 10^7 titer, or 7.08 log titer stock. The attenuation can be measured as the comparison of bacterial titer prior to modification of the bacterial nucleic acid to that after modification of the bacterial nucleic acid. The log of the ratio of the titer of unmodified bacteria to the titer of bacteria after modification represents the log attenuation (or simply the difference in log titer of the two). For example, if an unmodified bacterial titer measures $1.2x10^7$ and a modified bacterial titer measures $4.3x10^2$, the resulting level of attenuation is 4.45 log. For embodiments of the invention, the desired amount of attenuation can range from a two-fold reduction to much greater levels of attenuation, including a level where essentially no proliferation is observed, depending on the desired level of safety and the intended application of the bacteria. A two-fold attenuation in replication would be observed if for a given dilution, there are half as many in the population of bacteria where the nucleic acid is modified as there are in an unmodified

population of the bacteria (about 0.3 log attenuation). In some embodiments, the attenuation in proliferation is at least about 0.3 log, about 1 log, about 2 log, about 3 log, about 4 log about 5 log, about 6 log, or at least about 8 log. In some embodiments, the attenuation is in the range of about 0.3 to > 10 log, about 2 to >10 log, about 4 to >10 log, about 6 to >10 log, about 0.3-8 log, about 0.3-7 log, about 0.3-6 log, about 0.3-5 log, about 0.3-4 log, about 0.3-3 log, about 0.3-2 log, about 0.3-1 log, about 1-5 log, or about 2-5 log.. In some embodiments, the attenuation is in the range of about 1 to >10 log, 1-8 log, 1-6 log, also about 2-6 log, also about 2-5 log, also about 3-5 log. In one embodiment of the invention, the attenuation results in essentially complete inactivation (e.g. where no colonies are observed to the limit of detection), wherein the bacterial gene expression is sufficiently active. Such a population of bacteria can be achieved by titrating the concentration of the agent used to modify the bacterial nucleic acid to find the lowest concentration at which no colonies or plaques are observed at the limit of detection.

[0165] It is also possible to assess the attenuation in terms of biological effects of the *B. anthracis*. For example, the pathogenicity of a strain can be assessed by measurement of the median lethality (LD_{50}) in mice or other vertebrates. The LD_{50} is the amount (e.g. CFU or number of organisms in the case of non-proliferating bacteria) of bacteria injected into the vertebrate that would result in the death of half of the population of the vertebrate. The LD_{50} values can be compared for modified and unmodified bacteria as a measure of the amount of attenuation. For example, if an unmodified population of bacteria has an LD_{50} of 10^3 bacteria and the population of bacteria in which the nucleic acid has been modified has an LD_{50} of 10^5 bacteria, the bacteria has been attenuated so that its LD_{50} is increased 100-fold, or by 2 log. In some embodiments, the LD_{50} is 2-fold to 1000-fold higher. In some embodiments, an attenuated strain is used that already has a relatively high LD_{50}. In such cases, the increase in LD_{50} of the modified bacteria is limited by how much material can be infused without causing harm. For example, the LD_{50} of a heat killed organism would not be much higher than about 1-5 x 10^9 simply because of the loading of biological material into the mice and/or the inflammatory reaction to the bacterial wall components. The degree of attenuation may also be measured qualitatively by other biological effects, such as the extent of tissue pathology or serum liver enzyme levels. Typically, alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin, and billirubin levels in the serum are determined at a clinical laboratory for mice injected with bacteria of the present invention. Comparisons of these effects in mice or other

vertebrates would be made for unmodified and modified bacteria as a way to assess the attenuation of the bacteria. In addition to measuring the effects of the bacteria on the tissues, the amount of viable bacteria that can be recovered from infected tissues such as liver or spleen as a function of time could also be used as a measure of attenuation by comparing these values in mice injected with unmodified vs. modified strains of *Bacillus anthracis*.

2. Protein expression by *B. anthracis* attenuated for proliferation

[0166] In some embodiments, the modification of the nucleic acid of the bacteria, in addition to attenuating proliferation of the bacteria, is controlled so that bacterial gene expression is substantially unaffected. To be substantially unaffected, the bacterial gene expression need not be completely active upon modification of the nucleic acid. It is only necessary that in a population of bacteria in which the nucleic acid is modified to attenuate replication, bacterial gene expression is sufficiently active to provide an adequate level of expression of the desired protein by the bacterium. An adequate level of expression depends to some extent on the intended use of the bacteria. For example, if the bacterium contains a particular antigen that is to be used as a vaccine, adequate expression would be determined as the minimum level of expression that provides an effective protective or therapeutic immune response to the vaccine. The bacterial gene expression can also be assessed by both *in vitro* and *in vivo* methods in order to assess whether such a vaccine might provide an effective immune response. In general, a population of bacteria in which the nucleic acid has been modified can be compared to an unmodified population of the bacterium with respect to a particular antigen.

[0167] One possibility is to measure the presentation of the antigen of interest by an antigen presenting cell that has been mixed with a population of the bacteria. The bacteria may be mixed with a suitable antigen presenting cell or cell line, for example a dendritic cell, and the antigen presentation by the dendritic cell to a T cell that recognizes the antigen can be measured. If the bacteria are expressing the antigen at a sufficient level, it is processed into peptide fragments by the dendritic cells and presented in the context of MHC class I or class II to CD8+ or CD4+ T cells, respectively. For the purpose of detecting the presented antigen, a T cell clone or T cell line responsive to the particular antigen may be used. The T cell may also be a T cell hybridoma, where the T cell is immortalized by fusion with a cancer cell line. Such T cell hybridomas, T cell clones, or T cell lines can comprise either CD8+ or CD4+ T cells. The antigen presenting cell can present to either CD8+ or CD4+ T cells, depending on the pathway

by which the antigens are processed. CD8+ T cells recognize antigens in the context of MHC class I while CD4+ T cells recognize antigens in the context of MHC class II. The T cell is stimulated by the presented antigen through specific recognition by its T cell receptor, resulting in the production of certain proteins, such as IL-2 or interferon-gamma (IFN-gamma), that can be quantitatively measured (for example using an ELISA assay). Alternatively, a hybridoma can be designed to include a reporter gene, such as beta-galactosidase, that is activated upon stimulation of the T cell hybridoma by the presented antigens. The increase in the production of beta-galactosidase can be readily measured by its activity on a substrate, such as chlorophenolred-beta-D-galactopyranoside, which results in a color change. The color change can be directly measured as an indicator of specific antigen presentation. It is also possible to directly measure the expression of a particular protein by bacteria of the present invention. For example, a radioactively labeled amino acid can be added to a cell population and the amount of radioactivity incorporated into a particular protein can be determined. The proteins synthesized by the cell population can be isolated, for example by gel electrophoresis or capillary electrophoresis, identified as the protein of interest, e.g. by binding with an antibody-specific for the protein, and the amount of radioactivity can be quantitatively measured to assess the expression level of the particular protein. Alternatively, the proteins can be expressed without radioactivity and detected by various methods, such as an ELISA assay or by gel electrophoresis and Western blot with detection using an enzyme linked antibody or fluorescently labeled antibody.

[0168] While it is possible that the modification of the bacterial nucleic acid reduces the level of protein expression as compared to an unmodified bacterium, it is to be understood that this may still provide an effective vaccine. It is the combination of attenuation of proliferation with adequate protein expression that is important in some embodiments of the invention. The efficacy of a vaccine is generally related to the dose of antigen that can be delivered by the bacterium, and in some instances, some level of active gene expression by the bacteria is necessary. The attenuation of replication of the bacteria may be several log while the bacterial gene expression is still sufficiently maintained. If the same dose of an attenuated bacterium is compared to that of an unmodified bacterium, the resulting antigen expression (as assessed by the methods discussed above) in the attenuated bacteria population is at least about 1%, about 5%, about 10%, about 25%, about 50%, about 75% or at least about 90% of the antigen

expression in the unmodified bacteria population. Since there may be several log attenuation in replication, in some embodiments the dose of the modified bacteria may be safely increased by up to several log, resulting in an equivalent or greater amount of the antigen presented by the attenuated bacteria relative to unmodified bacteria upon vaccination.

3. Nucleic acid modification of *B. anthracis* genome

[0169] The nucleic acid of a population of bacteria can be modified by a variety of methods to attenuate the bacteria for proliferation.

[0170] The nucleic acid of the bacteria can be modified by physical means, e.g. irradiation with ultraviolet light or ionizing radiation. Ionizing radiation, such as x-rays or gamma-rays, may be used to cause single-strand or double-strand breaks in the nucleic acid. Ultraviolet radiation may be used to cause pyrimidine dimers in the nucleic acid. The appropriate dose of radiation is determined by assessing the effects of the radiation on replication and protein expression as detailed above. In some embodiments, radiation is used which causes DNA damage.

[0171] The nucleic acid of the bacteria can also be modified by chemical means, e.g. by reaction with a nucleic acid targeted compound (also referred to herein as a "nucleic acid targeting compound"). In some embodiments, the bacteria are treated with a nucleic acid targeted compound that can modify the nucleic acid such that the proliferation of the bacteria is attenuated. In some embodiments, the bacteria are treated with a nucleic acid targeted compound that can modify the nucleic acid such that the proliferation of the bacteria is attenuated, wherein the bacterial population is still able to express a desired protein antigen to a degree sufficient to elicit an immune response. The nucleic acid targeted compound is not limited to a particular mechanism of modifying the nucleic acid. Such compounds modify the nucleic acid either by reacting directly with the nucleic acid (i.e. all or some portion of the compound covalently binds to the nucleic acid), or by indirectly causing the modification of the nucleic acid (e.g. by causing oxygen damage via generation of singlet oxygen or oxygen radicals, by generating radicals of the compound that cause damage, or by other mechanisms of reduction or oxidation of the nucleic acid). Enediynes are an example of a class of compounds that form radical species that result in the cleavage of DNA double strands (Nicolaou et al., Proc. Natl. Acad. Sci. USA, 90:5881-5888 (1993)). Compounds that react directly with the nucleic acid may react upon activation of the compound, for example upon radiation of the compound. Compounds that react indirectly to

cause modification of the nucleic acid may require similar activation to generate either an activated species of the compound or to generate some other active species. While not being limited to the means for activation of nucleic acid targeted compounds, one embodiment of the invention includes the use of photoactivated compounds that either react directly with the nucleic acid or that generate a reactive species such as a reactive oxygen species (e.g. singlet oxygen) which then reacts with the nucleic acid.

[0172] The nucleic acid targeted compounds preferentially modify nucleic acids without significantly modifying other components of a biological sample. Such compounds provide adequate modification of the nucleic acid without significantly altering or damaging cell membranes, proteins, and lipids. Such compounds may modify these other cell components to some degree that is not significant. These cell components such as cell membranes, proteins and lipids are not significantly altered if their biological function is sufficiently maintained. In the case of treating a bacterium with a nucleic acid targeted compound, the nucleic acid modification is such that the replication of the bacteria is attenuated while the cell membranes, proteins and lipids of the bacteria are essentially unaffected such that bacterial gene expression is active (e.g. the enzymes required for this are not significantly affected), and the surface of the bacteria maintains essentially the same antigenicity as a bacterium that has not been treated with the compound. As a result, such compounds are useful in preparing an inactivated bacterium for use as a vaccine since the proliferation of the bacterium is sufficiently attenuated while maintaining sufficient antigenicity or immunogenicity to be useful as a vaccine. Because the compounds specifically modify nucleic acids, the modification can be controlled to a desired level so that replication is attenuated while maintaining a sufficient level of protein expression. The modification can be controlled by varying the parameters of the reaction, such as compound concentration, reaction media, controlling compound activation factors such as light dose or pH, or controlling compounds that cause oxygen damage by controlling the oxygen concentration (either physically, e.g. by degassing, or chemically, by use of oxygen or radical scavengers). A nucleic acid targeted compound is any compound that has a tendency to preferentially bind nucleic acid, i.e. has a measurable affinity for nucleic acid. Such compounds have a stronger affinity for nucleic acids than for most other components of a biological sample, especially components such as proteins, enzymes, lipids and membranes. The nucleic acid targeting provides specificity for the modification of nucleic acids without significantly affecting other

components of the biological sample, such as the machinery for gene transcription and protein translation.

[0173] Compounds can be targeted to nucleic acids in a number of modes. Compounds which bind by any of the following modes or combinations of them are considered nucleic acid targeted compounds. Intercalation, minor groove binding, major groove binding, electrostatic binding (e.g. phosphate backbone binding), and sequence-specific binding (via sequence recognition in the major or minor groove) are all non-covalent modes of binding to nucleic acids. Compounds that include one or more of these modes of binding will have a high affinity for nucleic acids. While the invention is not limited to the following compounds, some examples of compounds having these modes of binding to nucleic acid are as follows: intercalators are exemplified by acridines, acridones, proflavin, acriflavine, actinomycins, anthracyclinones, beta-rhodomycin A, daunamycin, thiaxanthenones, miracil D, anthramycin, mitomycin, echinomycin, quinomycin, triostin, diacridines, ellipticene (including dimers, trimers and analogs), norphilin A, fluorenes and flourenones, fluorenodiamines, quinacrine, benzacridines, phenazines, phenanthradines, phenothiazines, chlorpromazine, phenoxazines, benzothiazoles, xanthenes and thio-xanthenes, anthraquinones, anthrapyrazoles, benzothiopyranoindoles, 3,4-benzpyrene, benzopyrene diol epoxidie, 1-pyrenyloxirane, benzanthracene-5,6-oxide, benzodipyrones, benzothiazoles, quinolones, chloroquine, quinine, phenylquinoline carboxamides, furocoumarins (e.g. psoralens, isopsoralens, and sulfur analogs thereof), ethidium salts, propidium, coralyne, ellipticine cation and derivatives, polycyclic hydrocarbons and their oxirane derivatives, and echinimycin; minor groove binders are exemplified by distamycin, mitomycin, netropsin, other lexitropsins, Hoechst 33258 and other Hoechst dyes, DAPI (4',6'-diamidine-2-phenylindole), berenil, and triarylmethane dyes; major groove binders are exemplified by aflatoxins; electrostatic binders are exemplified by spermine, spermidine, and other polyamines; and sequence-specific binders are exemplified by nucleic acids or analogues which bind by such sequence-specific interactions as triple helix formation, D-loop formation, and direct base pairing to single stranded targets. Other sequence-specific binding compounds include poly pyrrole compounds, poly pyrrrole imidazole compounds, cyclopropylpyrroloindole compounds and related minor groove binding compounds (Wemmer, Nature Structural Biology, 5:169-171 (1998), Wurtz et al., Chemistry & Biology 7(3):153-161 (2000), Anthoney et al., Am. J. Pharmacogenomics 1:67-81 (2001)).

[0174] In addition to targeting nucleic acids, some of the compounds are also able to react with the nucleic acid, resulting in covalent binding to the nucleic acid. Nucleic acid alkylators are a class of compounds that can react covalently with nucleic acid and include, but are not limited to, mustards (e.g. mono or bis haloethylamine groups, and mono haloethylsulfide groups), mustard equivalents (e.g. epoxides, alpha-halo ketones) and mustard intermediates (e.g. aziridines, aziridiniums and their sulfur analogs), methanesulphonate esters, and nitroso ureas. The nucleic acid alkylators typically react with a nucleophilic group on the nucleic acid. It is the combination of the nucleic acid alkylating activity and the nucleic acid targeting ability of these compounds that gives them the ability to covalently react specifically with nucleic acids, providing the desired modification of the nucleic acid of bacteria for use in the present invention. The specificity of these compounds may be further enhanced by the use of a quencher that will not enter the bacteria. Such a quencher will quench reactions with the surface of the bacteria while still allowing the nucleic acid targeted compounds to react with the bacterial nucleic acid. A discussion of such quenching can be found in US Patent number 6,270,952, the disclosure of which is hereby incorporated by reference. The modification of the bacterial nucleic acid can be controlled by adjusting the compound concentration and reaction conditions. The appropriate concentration and reaction conditions are determined by assessing their effects on replication and protein expression as detailed above. The compounds used in the present invention are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1 nM to 10 μM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM. A discussion of nucleic acid targeted, nucleic acid reactive compounds for specific reaction with nucleic acids, in particular bacterial nucleic acids, can be found in U.S. Pat. Nos. 6,143,490 and 6,093,725, the disclosures of which are hereby incorporated by reference.

[0175] The nucleic acid can be modified by using a nucleic acid targeted compound that requires activation with radiation in order to cause the nucleic acid modification. Such compounds are targeted to nucleic acids as discussed above. These compounds include, but are not limited to, acridines, acridones, anthyrl derivatives, alloxazines (e.g. riboflavin), benzotriazole derivatives, planar aromatic diazo derivatives, planar aromatic cyano derivatives, toluidines, flavines, phenothiazines (e.g. methylene blue), furocoumarins, angelicins, psoralens, sulfur analogs of psoralens, quinolones, quinolines, quinoxalines, napthyridines, fluoroquinolones, anthraquinones, and anthracenes. Many of these compounds are used as DNA

photocleavage agents (Da Ros et al., Current Pharmaceutical Design 7:1781 (2001)). While the invention is not limited to the method of activation of the nucleic acid targeted compounds, typically, the compounds can be activated with light of particular wavelengths. The effective wavelength of light depends on the nature of the compound and can range anywhere from approximately 200 to 1200 nm. For some of these compounds, activation causes modification of the nucleic acid without direct binding of the compound to the nucleic acid, for example by generating reactive oxygen species in the vicinity of the nucleic acid. For some of these compounds, activation results in binding of the compound directly to the nucleic acid (i.e. the compound binds covalently). Some of these compounds can react with the nucleic acid to form an interstrand crosslink. Psoralens are an example of a class of compounds that crosslink nucleic acids. These compounds are typically activated with UVA light (320-400 nm). Psoralen compounds for use in the present invention are exemplified in U.S. Pat. Nos. 6,133,460 and 5,593,823, the disclosures of which are hereby incorporated by reference. Again, it is the combination of nucleic acid targeting and the ability to modify the nucleic acid upon activation that provide specific reactivity with nucleic acids. The modification of the bacterial nucleic acid can be controlled by adjusting the compound concentration, reaction conditions and light dose. The appropriate concentration and light dose are determined by assessing their effects on replication and protein expression as detailed above. In addition to compound concentration and level of light exposure, the reaction is affected by the conditions under which the sample is dosed with UVA light. For example, the required overall concentration for irradiating a population of bacteria in a buffered media is going to vary from a population that is cultured in a growth media (e.g. BHI, Tripticase Soy Broth). The photoreaction may be affected by the contents of the growth media, which may interact with the psoralen, thereby requiring a higher overall concentration of the psoralen. In addition, the effective dosing of the bacteria may depend on the growth phase of the organism and the presence or absence of compound during the growth phase. In one embodiment, the population of bacteria comprises growth media during the psoralen UVA treatment. In one embodiment, the psoralen is added to the population of bacteria, the population is cultured to grow the bacteria in the presence of psoralen and growth media, and the UVA treatment is performed at some point in the growth phase of the bacteria. In some embodiments, the population is grown to an OD of about 0.5 to about 1 (about 1×10^7 to about 1×10^9 CFU/mL) in the presence of the psoralen prior to irradiation with an appropriate

dose of UVA light. In some embodiments, the population is grown up to an OD of about 1 (1 x 10^9 CFU/mL) or above in the presence of the psoralen prior to irradiation with an appropriate dose of UVA light. Optionally, the population is grown up to a late log phase of an OD of about 1. In some embodiments, the population is grown up to about 1 x 10^{10} CFU/mL in the presence of psoralen, and then treated with UVA light. In some embodiments, the population is grown up to late log phase in the presence of psoralen and then concentrated to a concentration of up to about 1 x 10^{10} CFU/mL, and then treated with UVA light. Psoralen compounds are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1 nM to 10 μM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 100 J/cm^2, also about 0.1-20 J/cm^2, or about 0.5-10 J/cm^2, 0.5-6 J/cm^2 or about 2-6 J/cm^2. In one embodiment, the bacteria is treated in the presence of growth media at psoralen concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. In one embodiment, the bacteria treated in the presence of growth media is grown to an OD of 0.5-1 in the presence of psoralen at concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. Following the growth to an OD of 0.5-1, the bacteria population is irradiated with UVA light at a dose ranging from about 0.1 - 100 J/cm^2, also about 0.1-20 J/cm^2, or about 0.5-10 J/cm^2, 0.5-6 J/cm^2 or about 2-6 J/ cm^2.

[0176] As with any bacteria of the invention, the modification of the DNA of the repair deficient (e.g. *uvrAB* or *uvrABC* deficient) *B. anthracis* with psoralen can be controlled by adjusting the compound concentration, reaction conditions and light dose. The appropriate concentration, reaction conditions and light dose are determined by assessing their effects on replication and protein expression as detailed above. The use of repair deficient mutants provides an additional level of control of proliferation while maintaining adequate protein expression such that the parameters of concentration, reaction conditions and light dose can be adjusted over a wider range of conditions to provide a suitable population of bacteria. For example, there is a broader range of nucleic acid modification density over which proliferation can be completely inhibited without significantly affecting protein expression. The minimum level of modification required to completely inhibit repair deficient strains is much less than for non-repair deficient strains. As a result, the modification level can be higher than the minimum level required to stop proliferation (ensuring complete inactivation) yet still be below a level that

is detrimental to protein expression. Thus, while the invention is effective for non-repair deficient strains, *uvrAB* or *uvrABC* deficient strains provide greater flexibility in preparing a desirable population of bacteria that would be effective as a vaccine. Psoralen compounds are effective at concentrations of about 10 pM to 10 mM, also about 100 pM to 1 mM, also about 1 nM to 10 μM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 100 J/cm^2, also about 0.1-20 J/cm^2, also about 0.5-10 J/cm^2, or about 0.5-6 J/cm^2 or about 2-6 J/cm^2. In one embodiment, the bacterium is treated in the presence of growth media at psoralen concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. In one embodiment, the bacterium treated in the presence of growth media is grown to an OD of 0.5-1 in the presence of psoralen at concentrations of about 10 pM to 10 mM, also about 1-5000 nM, also about 1-500 nM, also about 5-500 nM, or about 10-400 nM. Following the growth to an OD of 0.5-1, the bacterial population is irradiated with UVA light at a dose ranging from about 0.1 - 100 J/cm^2, also about 0.1-20 J/cm^2, or about 0.5-10 J/cm^2, 0.5-6 J/cm^2 or about 2-6 J/ cm^2.

[0177] In order to generate primarily psoralen crosslinks in any *B. anthracis* bacterium, particularly *uvrAB* or *uvrABC* deficient mutant *B. anthracis*, it is possible to dose the psoralen and UVA light initially to form adducts and follow this with a second dose of UVA light alone to convert some or most of the monoadducts to crosslinks. The psoralen photochemistry is such that absorption of a photon of appropriate energy will first form a monoadduct. Absorption of an additional photon will convert this monoadduct to a crosslink when a furan side monoadduct is appropriately situated in the DNA double helix (Tessman et al., Biochemistry 24:1669-1676 (1985)). The sample can be dosed with a lower UVA dose at a desired concentration of psoralen and the unreacted psoralen can be removed, e.g. by washing, dialysis or ultrafiltration of the bacteria. The bacteria containing psoralen adducts (monoadducts and crosslinks) can be further dosed with UVA light to convert some or most of the monoadducts to crosslinks without resulting in significant additional adducts to the bacteria. This allows for the controlled addition of a low number of psoralen adducts with the initial light dose, then converting a substantial number of any monoadducts to crosslink with the second dose. This provides for modification of the bacterial genome at sufficiently low levels wherein a majority of the adducts formed will be crosslinks. This is particularly effective for blocking replication with *uvrAB* or *uvrABC* deficient mutants. In such embodiments, psoralen compounds are effective at concentrations of about 10

pM to 10 mM, also about 100 pM to 1 mM, also about 1-500 nM, also about 1-200 nM or about 1-100 nM, with the UVA light dose ranging from about 0.1 - 10 J/cm^2, also about 0.1-2 J/cm^2, or about 0.5-2 J/cm^2. Following removal of most of the unreacted psoralen by washing, dialysis or ultrafiltration of the bacteria, the bacteria may be dosed with UVA light ranging from 0.1 - 100 J/cm^2, also about 0.1-20 J/cm^2, or about 0.5-10 J/cm^2 or about 2-6 J/cm^2.

[0178] In some preferred embodiments, the nucleic acid targeted compound used to modify the nucleic acid of the *Bacillus anthracis* strain is an alkylator such as β-alanine, N-(acridin-9-yl), 2-[bis(2-chloroethyl)amino]ethyl ester. In other preferred embodiments, the nucleic acid targeted compound used to modify the nucleic acid of the *Bacillus anthracis* strain is a psoralen compound (e.g., 4'-(4-amino-2-oxa)butyl-4,5',8-trimethylpsoralen, also referred to herein as "S-59") activated by UVA irradiation.

[0179] Additional information regarding the modification of microbes such as *Bacillus anthracis* can be found in U.S. Provisional Application No. 60/446,051, filed February 6, 2003; U.S. Provisional Application No. 60/449,153, filed February 21, 2003; U.S. Provisional Application No. 60/490,089, filed July 24, 2003; U.S. Provisional Application No. 60/511,869, filed October 15, 2003; U.S. Serial No. (U.S. Patent Publication No. 2004/0197343 A1), filed February 6, 2004; and U.S. Patent Application Serial No. 10/883,599, filed June 30, 2004, both of which are incorporated by reference herein in their entirety.

[0180] The increased sensitivity of a *B. anthracis uvrAB* deletion mutant to psoralen/UVA treatment is illustrated in Example 3 and 14, below.

II. PHARMACEUTICAL COMPOSITIONS, IMMUNOGENIC COMPOSITIONS, VACCINES, AND FORMULATIONS

[0181] Compositions comprising any of the modified *B. anthracis* strains described herein are also provided. In some embodiments, the compositions are pharmaceutical compositions. In some embodiments, the compositions are immunogenic compositions. The pharmaceutical compositions optionally comprise a pharmaceutically acceptable carrier or adjuvant. In some embodiments, the compositions are vaccine compositions (i.e., vaccines). The vaccine compositions optionally comprise a pharmaceutically acceptable carrier or adjuvant.

[0182] For instance, in some embodiments, the invention provides an immunogenic composition comprising a modified *B. anthracis* bacterium and/or strain described herein.

[0183] In some embodiments, the invention provides a pharmaceutical composition comprising a modified *B. anthracis* bacterium and/or strain described herein and a pharmaceutically acceptable carrier.

[0184] In some embodiments, the invention provides a vaccine comprising a modified *B. anthracis* bacterium and/or strain described herein. In some embodiments, the vaccine further comprises an adjuvant.

[0185] The vaccine compositions of the present invention can be used to stimulate an immune response in an individual. The formulations can be administered to an individual by a variety of administration routes. Methods of administration of such a vaccine composition are known in the art, and include oral, nasal, intraveneous, intradermal, intraperitoneal, intramuscular, intralymphatic and subcutaneous routes of administration, as well any other route that is relevant for infectious disease.

[0186] The vaccine compositions may further comprise additional components known in the art to improve the immune response to a vaccine, such as adjuvants, T cell co-stimulatory molecules, or antibodies, such as anti-CTLA4. The invention also includes medicaments comprising the pharmaceutical compositions of the invention. An individual to be treated with such vaccines, is any vertebrate, preferably a mammal, including domestic animals, sport animals, and primates, including humans.

[0187] The vaccine is typically administered as a prophylactic.

[0188] Vaccine formulations are known in the art. Known vaccine formulations can include one or more possible additives, such as carriers, preservatives, stabilizers, adjuvants, antibiotics, and other substances. Preservatives, such as thimerosal or 2-phenoxy ethanol, can be added to slow or stop the growth of bacteria or fungi resulting from inadvertent contamination, especially as might occur with vaccine vials intended for multiple uses or doses. Stabilizers, such as lactose or monosodium glutamate (MSG), can be added to stabilize the vaccine formulation against a variety of conditions, such as temperature variations or a freeze-drying process. Adjuvants, such as aluminum hydroxide or aluminum phosphate, are optionally added to increase the ability of the vaccine to trigger, enhance, or prolong an immune response. Additional materials, such as cytokines, chemokines, and bacterial nucleic acid sequences, like CpG, are also potential vaccine adjuvants. Antibiotics, such as neomycin and streptomycin, are optionally added to prevent the potentially harmful growth of germs. Vaccines may also include

a suspending fluid such as sterile water or saline. Vaccines may also contain small amounts of residual materials from the manufacturing process, such as cell or bacterial proteins, egg proteins (from vaccines that are produced in eggs), DNA or RNA, or formaldehyde from a toxoiding process. Formulations may be resuspended or diluted in a suitable diluent such as sterile water, saline, isotonic buffered saline (e.g. phosphate buffered to physiological pH), or other suitable diluent.

[0189] The modified *B. anthracis* vaccine (or other composition comprising the modified *B. anthracis*) is optionally administered to a host in a physiologically acceptable carrier. Optionally, the vaccine formulation further comprises an adjuvant. Useful carriers known to those of ordinary skill in the art include, e.g., citrate-bicarbonate buffer, buffered water, 0.4% saline, and the like.

[0190] In some embodiments, the vaccine compositions are prepared as liquid suspensions. In other embodiments, the vaccine compositions comprising the *B. anthracis* strains are lyophilized (i.e., freeze-dried). The lyophilized preparation can then be combined with a sterile solution (e.g., citrate-bicarbonate buffer, buffered water, 0.4% saline, or the like) prior to administration.

III. IMMUNOGENICITY AND IN VIVO EFFICACY

[0191] The immunogenicity of a modified (e.g., mutant) *B. anthracis* bacteria described herein or a composition comprising the modified *B. anthracis* bacteria can be readily evaluated using a variety of assays known to those skilled in the art. For instance, as an initial step in the evaluation of a potential vaccine candidate, expression of the relevant antigens from the mutant can be assessed, typically by Western blot analysis (see e.g., Example 9, below). Mass spectrometry can also be used to assess expression of the desired proteins by the *B. anthracis* mutant (Lenz et al., Proc. Natl. Acad. Sci. USA, 100:12432-12437 (2003)). Assays well known to those in the art such as the Elispot assay (see, e.g., Example 10, below) and intracellular cytokine staining assay (ICS) can be used to assess the immunogenicity of the candidate vaccines. T-cell proliferation following immunization also provides an indication of immunogenicity (see, e.g., Example 11, below). Alternatively, the cytokine expression of stimulated spleen cells can be evaluated following immunization (e.g., immunization of a model organism, such as a mouse model) to determine the immunogenicity of the strain or composition

(see, e.g., Example 11). In addition, the cytotoxic activity of antigen-specific T cells produced using the *B. anthracis* can also be assessed, either *in vivo* or *in vitro*, using methods familiar to those in the art. The levels of antibodies produced in response to immunization of a mouse model or other host with the *B. anthracis* can also be assessed to evaluate the immunogenicity of the vaccine (see, e.g., Example 10, below). The ability of the generated antibodies to neutralize a *Bacillus anthracis* toxin can also be tested (see, e.g., Example 10).

[0192] The efficacy of the vaccines can be evaluated in an individual, for example in a mouse. A mouse model is recognized as a model for efficacy in humans and is useful in assessing and defining the vaccines of the present invention. The mouse model is used to demonstrate the potential for the effectiveness of the vaccines in any individual. Vaccines can be evaluated for their ability to provide either a prophylactic or therapeutic effect against a particular disease. For example, in the case of infectious diseases, a population of mice can be vaccinated with a desired amount of the *B. anthracis* vaccine. The mice can be subsequently infected with the infectious *B. anthracis* agent and assessed for protection against infection. The progression of the infectious disease can be observed relative to a control population (either non vaccinated or vaccinated with vehicle only). For instance, the protection against spore and lethal toxin afforded by the modified (e.g., mutant) *B. anthracis* bacterium or a vaccine comprising the modified bacterium can be evaluated using a mouse model system (see, e.g., Example 12, below).

[0193] In some embodiments, the present invention provides modified *Bacillus anthracis* bacteria, and compositions thereof (e.g., vaccine compositions), which are capable of generating an immune response upon administration to a host. In some embodiments, the immune response is a response which protects the host from a disease related to infection by *Bacillus anthracis*, such as anthrax. In some embodiments, the immune response comprises a CD4+ immune response, a CD8+ immune response, or both a CD4+ and CD8+ immune response. In some embodiments, the immune response comprises an immune response specific to lethal factor (LF), edema factor (EF), protective antigen (PA), capsule, and/or whole bacteria. In some embodiments, the bacteria and/or compositions described are capable of protecting a host against spore and/or lethal toxin challenge.

IV. METHODS OF USE

[0194] A variety of methods of using the modified *B. anthracis* strains, vaccines, and pharmaceutical compositions described herein are provided by the present invention. For instance, methods of using any of the modified *B. anthracis* strains, vaccines, and pharmaceutical compositions described herein to induce immune responses and/or to prevent disease (i.e., disease due to infection of the host by *B. anthracis*) are provided. Methods of using the modified *B. anthracis* strains to prepare vaccines and other compositions are also provided. The prevention of disease (also referred to herein as protection of a host from disease) provided by the vaccine compositions described herein need not necessarily be complete in order for the vaccine compositions to be useful.

[0195] In another aspect, the invention provides a method of protecting a host from a disease comprising administering to the host an effective amount of a compostion comprising a modified *Bacillus anthracis* bacterium and/or a strain described herein. In some embodiments, the disease is a disease associated with infection of a host by *Bacillus anthracis*. In some embodiments, the disease is anthrax.

[0196] As used herein, the terms "preventing" disease or "protecting a host" from disease (used interchangeably herein) encompass, but are not limited to, one or more of the following: stopping, deferring, hindering, slowing, retarding, and/or postponing the infection by *Bacillus anthracis* of a host; stopping, deferring, hindering, slowing, retarding, and/or postponing progression of an infection by *Bacillus anthracis* of a host; stopping, deferring, hindering, slowing, retarding, and/or postponing the onset or progression of a disease associated with infection of a host by *Bacillus anthracis*; and stabilizing the progression of a disease associated with infection of a host by *Bacillus anthracis*.

[0197] In some embodiments, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a modified *Bacillus anthracis* bacterium and/or strain described herein. In some embodiments, the immune response to *Bacillus anthracis* comprises an immune response to one or more *Bacillus anthracis* antigens (e.g., protective antigen, lethal factor and/or edema factor), capsule, and/or whole bacteria.

[0198] In some embodiments, the immune response which is induced by the compositions described herein is a humoral response. In other embodiments, the immune

response which is induced is a cellular immune response. In some embodiments, the immune response is B-cell response. In some embodiments, the immune response is a T-cell response (either a CD4+ T-cell response, a CD8+ T-cell response, or both). In some embodiments, the immune response comprises both cellular and humoral immune responses. In some embodiments, the immune response comprises an antigen-specific immune response.

[0199] In some embodiments, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of an asporogenic *B. anthracis* strain that is attenuated for nucleic acid repair.

[0200] The invention also provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of an asporogenic *B. anthracis* strain that is attenuated for nucleic acid repair.

[0201] In addition, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a heterologous expression cassette comprising a sequence encoding protective antigen, wherein the protective antigen coding sequence is operably linked to an inducible promoter.

[0202] The invention further provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a heterologous expression cassette comprising a sequence encoding protective antigen, wherein the protective antigen coding sequence is operably linked to an inducible promoter.

[0203] In addition, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence.

[0204] The invention further provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a heterologous expression cassette that expresses protective antigen under the control of an SOS regulatory sequence.

[0205] The invention further provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain that is defective with respect to RecA (e.g., a conditional or repressible *recA* mutant).

[0206] The invention also provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain that is defective with respect to RecA (e.g., a conditional or repressible *recA* mutant).

[0207] Furthermore, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a temperature sensitive *recA* gene.

[0208] Furthermore, the invention provides a method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium of an asporogenic *B. anthracis* strain attenuated for nucleic acid repair, comprising a temperature sensitive *recA* gene and a mutation in the *uvrAB* gene.

[0209] The invention also provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of a *B. anthracis* strain comprising a temperature sensitive *recA* gene.

[0210] The invention also provides a method of protecting a host from a disease, comprising administering to the host an effective amount of a composition comprising a bacterium of an asporogenic *B. anthracis* strain attenuated for nucleic acid repair, comprising a temperature sensitive *recA* gene and a mutation in the *uvrAB* gene.

[0211] The delivery of the recombinant bacteria, or a composition comprising the strain, may be by any suitable method, such as intradermal, subcutaneous, intraperitoneal, intravenous, intramuscular, intralymphatic, oral or intranasal.

[0212] The compositions comprising the recombinant bacteria and an immunostimulatory agent may be administered to a host simultaneously, sequentially or separately. Examples of immunostimulatory agents include, but are not limited to IL-2, IL-12, GMCSF, IL-15, B7.1, B7.2, and B7-DC and IL-14.

[0213] The host in the methods described herein, is any vertebrate, preferably a mammal, including domestic animals, sport animals, and primates, including humans. The term "host" is used interchangeably herein with the term "subject."

[0214] The dosage of the pharmaceutical compositions, immunogenic compositions, or vaccines described herein that are given to the host will vary depending on the species of the host, the size of the host, and the condition or disease of the host. The dosage of the compositions will also depend on the frequency of administration of the compositions and the route of administration. The exact dosage is chosen by the individual physician in view of the patient to be treated.

[0215] In some embodiments, a single dose of the pharmaceutical composition, immunogenic composition, or vaccine comprising a modified (e.g., mutant) *Bacillus anthracis* described herein comprises from about 1 x 10^2 to about 1 x 10^{12} of the *Bacillus anthracis* organisms. In some embodiments, a single dose of the composition comprises from about 1 x 10^5 to about 1 x 10^{11} organisms. In another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^6 to about 1 x 10^{11} of the *Bacillus anthracis* organisms. In still another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^6 to about 1 x 10^{10} of the *Bacillus anthracis* organisms. In still another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^7 to about 1 x 10^{10} of the *Bacillus anthracis* organisms. In still another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^7 to about 1 x 10^9 of the *Bacillus anthracis* organisms.

[0216] In some embodiments, a single dosage comprises at least about 1 x 10^2 *Bacillus anthracis* organisms. In some embodiments, a single dose of the composition comprises at least about 1 x 10^5 organisms. In another embodiment, a single dose of the composition or vaccine comprises at least about 1 x 10^6 *Bacillus anthracis* organisms. In still another embodiment, a single dose of the composition or vaccine comprises at least about 1 x 10^7 of the *Bacillus anthracis* organisms.

[0217] In some embodiments, a single dose of the pharmaceutical composition, immunogenic composition, or vaccine comprising a modified (e.g., mutant and/or treated with a nucleic acid targeting compound) *Bacillus anthracis* described herein comprises from about 1 CFU/kg to about 1 x 10^{10} CFU/kg (CFU = colony forming units). In some embodiments, a single dose of the composition comprises from about 10 CFU/kg to about 1 x 10^9 CFU/kg. In another

embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^2 CFU/kg to about 1 x 10^8 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^3 CFU/kg to about 1 x 10^8 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from about 1 x 10^4 CFU/kg to about 1 x 10^7 CFU/kg. In some embodiments, a single dose of the composition comprises at least about 1 CFU/kg. In some embodiments, a single dose of the composition comprises at least about 10 CFU/kg. In another embodiment, a single dose of the composition or vaccine comprises at least about 1 x 10^2 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises at least about 1 x 10^3 CFU/kg. In still another embodiment, a single dose of the composition or vaccine comprises from at least about 1 x 10^4 CFU/kg. (In some embodiments, for instance, in some embodiments where the modified bacteria are attenuated for proliferation by a nucleic acid targeting compound, the CFU amounts may refer to CFU of the bacteria prior to the modification with the compound that attenuates the bacteria for proliferation.)

[0218] In some embodiments, the proper (i.e., effective) dosage amount for one host, such as human, may be extrapolated from the LD_{50} data for another host, such as a mouse, using methods known to those in the art.

[0219] In some embodiments, multiple adminstrations of the dosage unit are preferred, either in a single day or over the course of a week or month or year or years. In some embodiments, the dosage unit is administered every day for multiple days, or once a week for multiple weeks.

[0220] In another aspect, the invention provides the use of any modified *Bacillus anthracis* bacterium and/or strain described herein in the manufacture of a medicament for inducing an immune response in a host to *Bacillus anthracis*.

[0221] In still another aspect, the invention provides the use of any modified *Bacillus anthracis* bacterium and/or strain described herein in the manufacture of a medicament for protecting a host against a disease. In some embodiments, the disease is a disease associated with *Bacillus anthracis* infection (e.g., anthrax).

[0222] In addition to use directly in vaccines and other immunogenic compositions, the modified *B. anthracis* bacteria of the present invention provide reagents and methods for the expression, production, or biosynthesis of, e.g., enzymes and proteins, for use in, for example,

industrial, veterinary, medical, and/or diagnostic applications. For instance, in some embodiments, the modified bacteria may be used for the production of protective antigen, an antigen currently often used in current anthrax vaccines.

V. KITS

[0223] The invention further provides kits (or articles of manufacture) comprising the modified *Bacillus anthracis* strains and bacteria of the present invention.

[0224] In one aspect, the invention provides a kit comprising both (a) a composition comprising a modified *B. anthracis* strain and/or bacterium described herein, and (b) instructions for the use of the composition in the prevention or treatment of a disease in a host. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0225] In another aspect, the invention provides a kit comprising both (a) a composition comprising a modified *B. anthracis* strain and/or bacterium described herein; and (b) instructions for the administration of the composition to a host. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0226] In another aspect, the invention provides a kit comprising both (a) a composition comprising a modified *B. anthracis* strain and/or bacterium described herein; and (b) instructions for selecting a host to which the composition is to be administered. In some embodiments, the instructions are on a label. In other embodiments, the instructions are on an insert contained within the kit.

[0227] In some embodiments of each of the aforementioned aspects, the composition is a vaccine. In some embodiments of each of the aforementioned aspects, the *B. anthracis* is defective with respect to a DNA repair enzyme. In some embodiments of each of the aforementioned aspects, the nucleic acid of the *B. anthracis* has been modified by reaction with a nucleic acid targeted compound that reacts directly with the nucleic acid. In some embodiments, the *B. anthracis* has been S-59/UVA treated.

EXAMPLES

[0228] The following examples are provided to illustrate, but not to limit, the invention.

EXAMPLE 1

Bacterial Vaccines derived from nucleotide-excision repair (NER) mutants

[0229] The examples described herein illustrate the efficacy of vaccine compositions utilizing genomic inactivation through photochemical treatment of the recombinant delivery platform encoding antigens related to infectious and malignant disease. According to this composition, while the genomes are inactivated and cannot separate during replication, the transcriptional profile remains largely intact, thus resulting in antigen expression *de novo* in the vaccinated individual, and optimal induction of pathogen-specific immune responses, including CD8+ cytotoxic T cells (CTL). Furthermore, by utilizing a vaccine platform in this composition in which the DNA nucleotide excision repair (NER) machinery has been inactivated by any number of means, including by engineered genetic deletion, the sensitivity to photochemical inactivation in these mutants is dramatically increased.

[0230] As a result of the requirement of significantly fewer DNA cross-links to inactivate the DNA repair mutants, in the context of the population of bacterial genomes comprising a vaccine dose, the expression of any one gene will not be significantly affected, due to the low level of DNA crosslinking resulting in essentially no interruption of expression, at that given gene.

[0231] Thus, the overall utility of gene-based vaccines utilizing bacterial platforms derived from pathogens can be increased dramatically by combining photochemical inactivation with a vector defective in NER. While the inactivated vaccine cannot cause disease, it still retains its efficient ability to induce potent immunity, including T-cell mediated cellular immunity, specific for the vector-expressed heterologous antigens. Furthermore, the *uvrAB* mutation can be combined with any other attenuating mutation(s), in order to derive a safe and efficacious vaccine platform combining both photochemical and genetic attenuation.

[0232] Significantly, these compositions can be used as an approach for deriving a safe and efficacious vaccine derived from a selected bacterial pathogen, in order to protect against challenge with the wild-type pathogen in vaccinated individuals. According to this application, it is not feasible in many cases to derive a safe and efficacious vaccine that is derived from an attenuated viable form of the pathogen, as the possibility for reactivity and disease pathogenesis

in particular individuals receiving the vaccine remain high. While subunit or inactivated vaccines related to a selected bacterial pathogen might be safe, on the other hand, these vaccines are often not efficacious because they do not efficiently elicit the breadth, depth, and durability of pathogen-specific immune responses that are required to protect the vaccinated individual against challenge with the wild-type form of the said pathogen. Thus, it is well known in the art that there is a clear need for vaccine compositions that combine safety with an efficient ability to elicit the type of immune responses in vaccinated individuals that are protective.

[0233] As such, mutants in the nucleotide-excision repair (NER) pathway of *B. anthracis* provide a composition that can be used for safe and efficacious vaccines that elicit protection against challenge in immunized individuals with amounts of bacteria that are sufficient to cause disease in non-vaccinated individuals. NER is catalyzed by an ATP-dependent nuclease made of three subunits, known as the ABC excinuclease, and encoded by the genes *uvrA*, *uvrB*, and *uvrC*. Mutations in any one or more than one of the three *uvr* genes results in cells, including microbes of pathogenic organisms like *B. anthracis*, extremely sensitive to DNA modification, including photochemical inactivation utilizing psoralens and UVA light.

[0234] As an example, mutation of the *uvr* genes of *Bacillus anthracis* (*B. anthracis*), the etiological agent of Anthrax, is provided. The current acellular anthrax vaccines that are licensed for human use are based on sterile culture supernatants of attenuated *B. anthracis* adsorbed on alum hydroxide (U.S. vaccine), or precipitated with alum phosphate (U.K. vaccine). It is well known that these vaccines are rather weak, requiring at least six immunizations for protection as well as annual boosters.

[0235] In the composition described herein, the *uvrA*, *uvrB*, or *uvrC* genes, or any *B. anthracis* gene involved in NER, alone, or in any combination, is mutated such that a functional form of the protein is not expressed.

[0236] As an example, mutation in the *uvrA*, *uvrB*, or *uvrC* genes, or any *B. anthracis* gene involved in NER, can be performed, for example, by allelic exchange using the pKSV7 vector, as described in Camilli et al., *Molecular Microbiology*, 8:143-147 (1993). *B. anthracis* genes involved in NER are identified through a homology search with the genomes of related organisms in whose *uvr* genes are known. For example, the genome of *B. anthracis*, that is, the main chromosome and the two virulence plasmids can be compared with *Bacillus subtilis* (*B. subtilis*), a related bacterium from the same genera as *B. anthracis*. The genomic scaffold

representing the main chromosome of the Florida *B. anthracis* isolate (Read et. al. 2002. *Science 296*, 2028-2033) has a GenBank accession number of AAAC010000001. *B. subtilis* has a GenBank accession number of NC_000964. The *B. subtilis uvrA* gene encompasses nts. 3609064 to 3611997, and the *B. subtilis uvrB* gene encompasses nts. 3612005-3613990. A BLAST search was performed using the *B. subtilis uvrA* and *uvrB* coding sequences against the *B. anthracis* sequence. This analysis identified a region of 72% sequence identity in the genome of *B. anthracis* that corresponds to the *uvrA* and *uvrB* genes of this organism. The *B. anthracis uvrA* gene maps from 226021-228783, and bears 72% sequence homology to the *B. subtilis uvrA* gene (2082/2867 identical sequence homology alignment). The *B. anthracis uvrB* gene maps from 228864-230771, and bears 72% sequence homology to the *B. subtilis uvrB* gene (1401/1925 identical sequence homology alignment). Thus, the *B. anthracis uvrAB* genes include nts. 226021 to 230771 of the main chromosome of *B. anthracis*.

[0237] Deletion of the *B. anthracis uvrAB* genes, including nts. 226021 to 230771 of the main bacterial chromosome is accomplished according to the methods described in Camilli et al., *Molecular Microbiology*, 8:143-147 (1993) and as described for *Listeria monocytogenes* in Example 7 of U.S. Serial No. 10/773,618, filed February 6, 2004 (U.S. Patent Publication No. 2004/0197343 A1). Briefly, this region and approximately 1000 bps both upstream and downstream of the *B. anthracis* genome are amplified by PCR, and subsequently cloned into the pKSV7 allelic exchange plasmid vector. As an alternative, a *Bacillus* genera-specific or *B. anthracis*-specific temperature-sensitive (*ts*) replicon may be substituted for the *Listeria ts* replicon present in the pKSV7 allelic exchange plasmid vector. Using convenient restriction endonuclease recognition sites mapping specifically within the *uvrAB* region, any part of the *uvrA*, *uvrB*, or all of the *uvrAB* genes sequence are deleted.

[0238] Finally, the allelic exchange plasmid is introduced into *B. anthracis* and NER mutants are selected as described in Camilli et al., *Molecular Microbiology*, 8:143–147 (1993) and as described for *Listeria monocytogenes* in Example 7 of U.S. Patent Publication No. 2004/0197343 A1. Briefly, bacteria electroporated with the pKSV7-heterologous protein expression cassette plasmid are selected by plating on BHI agar media containing chloramphenicol (10 μg/ml), and incubated at the permissive temperature of 30°C. Single cross-over integration into the bacterial chromosome or deletion of the *uvrAB* genes from the bacterial chromosome is selected by passaging several individual colonies for multiple generations at the

non-permissive temperature of 41°C in media containing chloramphenicol. Finally, plasmid excision and curing (double cross-over) is achieved by passaging several individual colonies for multiple generations at the permissive temperature of 30°C in BHI media not containing chloramphenicol. Verification of integration of the heterologous protein expression cassette into the bacteria chromosome is verified by PCR, utilizing a primer pair that amplifies a region defined from within the heterologous protein expression cassette to the bacterial chromosome targeting sequence not contained in the pKSV7 plasmid vector construct, or utilizing a primer pair that hybridizes to the bacterial chromosome beyond the deleted region, and thus deletion of the targeted gene is observed by a PCR amplicon of decreased molecular weight, as compared to the parental strain. Any selected *B. anthracis* strain can be used as a parent strain for derivation of the NER-defective vaccine, including, for example, the following strains: Ames, Vollum, A1.a/10, A1.b/23, A2/29, A3.a/34, A3.b/57, A4/69, B/80, Δsterne, VN41Δ1, Dames, NNR1Δ1, and DNH1. An exemplary preparation of the *uvrAB* deletion mutant of the *Bacillus anthracis* Sterne strain is described below in Example 2.

[0239] Additionally, other attenuating mutations can be incorporated into the genome of the selected NER mutant *B. anthracis* strain, to enable vaccine compositions combining DNA modification, including photochemical inactivation, with genetic inactivation. Such *B. anthracis* vaccine compositions are able to induce immune responses against known correlates of anthrax immunity and protection, including lethal factor (LF), edema factor (EF), and protective antigen (PA). Additionally, since the expression profile of the NER mutant vaccine composition is not significantly affected, immune responses against other antigens, including those expressed from the two virulence plasmids pXO1 and pXO2 and the main chromosome are also induced.

[0240] The compositions described herein in some embodiments, using *B. anthracis* NER mutants as a component of vaccine, can be used in a prophylactic immunization against disease caused by cutaneous, gastrointestinal or respiratory infections.

EXAMPLE 2

Construction of a *Bacillus anthracis* Sterne Δ*uvrAB*.

[0241] The allelic exchange methods detailed in Camilli et al., *Molecular Micro.*, 8:143-147 (1993) and as described in U.S. Patent Publication No. 2004/0197343 A1, for alteration of

Listeria monocytogenes were used to modify the *Bacillus anthracis* Sterne strain. The virulence of this strain is attenuated ($pXO1^+$, $pXO2^-$). All of the TOPO vectors used here were derived from pCR®2.1-TOPO (Invitrogen, Carlsbad, CA).

[0242] The *uvrAB* gene from *Bacillus anthracis* was identified (Genbank accession number AE017040, *Bacillus anthracis* Ames strain, section 17 of 18 of the complete genome, *uvrAB* genes coding sequence: nts. 212613-217471) and a plasmid based on pKSV7 with the *uvrAB* gene deletion was constructed (pKSV7-*dl uvrAB*) using Splice Overlap Extension (SOE) PCR and the steps described below:

[0243] *Primary PCR reactions:* Approximately 1000 bps of sequence upstream and downstream from the *B. anthracis uvrAB* genes 5' and 3' ends, respectively, were amplified.

[0244] Template: *B. anthracis* Sterne genomic DNA

[0245] Primer pair 1: Amplification of region 1000 bp upstream from 5' end of *uvrB*. (Amplicon Size (bps): 1029)

[0246] Ba-225099F: 5'-CTGTGCTTTGCGAATGGAAAGAAGC (SEQ ID NO:6) (T_m: 74°C).

[0247] Ba-(3' uvrA-R +) 226109R: 5'-GTTTTCATTCATACACTTAGACAAGCGTTGGCTTTTGCACTTC (SEQ ID NO:7) (T_m: 120°C) (Underlined sequence is complementary to region downstream of *uvrA* carboxy terminus.) *or* Ba-226109R: 5'-GACAAGCGTTGGCTTTTGCACTTC (SEQ ID NO:8) (T_m: 72°C).

[0248] Primer pair 2: Amplification of region downstream from 3' end of *uvrA*. (Amplicon size (bps): 990)

[0249] Ba-(3' uvrA-R +) 230779F: 5'-CAAAAGCCAACGCTTGTCTAAGTGTATGAATGAAAACCGAGTGG (SEQ ID NO:9) (T_m: 126°C) (Underlined sequence is complementary to region upstream of *uvrB* amino terminus.) *or* Ba-230779F: 5'-AAGTGTATGAATGAAAACCGAGTGG (SEQ ID NO:10) (T_m: 70°C)

[0250] Ba-231769R: 5'-CATATAAAGGTTCCACAATTGCCTTTTC (SEQ ID NO:11) (T_m: 76°C)

[0251] *Secondary PCR reaction*: Fusion of primary PCR amplicons through SOE PCR, taking advantage of complementarity between reverse primer of pair 1 and the forward primer of pair 2. Results in precise deletion of *uvrAB* coding sequence: nts. 226110-230779=4670 bps.

[0252] Template: Cleaned primary PCR reactions

[0253] Primer pair: (Amplicon size (bps): 1973)

[0254] Ba-225118F: 5'-GAAGCAGAAATGAAGCCAATACTCAATC (SEQ ID NO:12) (T_m: 78°C)

[0255] Ba-231761R: 5'-GGTTCCACAATTGCCTTTTCAATAATC (SEQ ID NO:13) (T_m: 74°C)

[0256] *Construction:* Primary PCR reactions (3 temperature cycle) were performed using Vent DNA polymerase (NEB) and Sterne strain genomic DNA. Four primary PCR reactions were performed both with and without primers used for splice overlap extension (SOE). (If reactions containing Ba-(3' uvrA-R +) 226109R or Ba-(3' uvrA-R +) 226109R primers did not yield significant amplicon product, then these primers on amplicons from reactions with Ba-225099F/Ba-226109R or Ba-230779F/ Ba-231769R primer pairs were used.) The expected size of *anthracis* primary amplicons by 1% agarose gel (1029 bps and 990 bps) was verified. The reaction was cleaned with S6 columns (BioRad, Hercules, CA) or GeneClean (BIO 101, Irvine, CA).

[0257] The secondary PCR reaction was performed, utilizing approximately equal amounts of each primary reaction as template (ca. 5 µl) were performed. The expected size of the *Listeria* amplicon from secondary PCR reaction by 1% agarose gel (1973 bps) was verified.

[0258] The *anthracis dl uvrAB* amplicon was inserted into pCR2.1-Blunt II-TOPO vector. The plasmid pCR2.1-TOPO-*dl uvrAB* plasmid DNA was digested with *KpnI* and *PstI* and gel-purify 2033 bp fragment. The *KpnI*/*PstI* 2033 bp fragment was inserted into pKSV7 vector, that had been prepared by digestion with *KpnI* and *PstI* and treatment with CIAP (pKSV7-*dl uvrAB*). The fidelity of *dl uvrAB* sequence in pKSV7-*dl uvrAB* was verified.

[0259] The *uvrAB* genes were deleted from *B. anthracis* Sterne by allelic exchange with pKSV7-*dl uvrAB* plasmid. The plasmid pKSV7-*dl uvrAB* was introduced into the *B. anthracis* Sterne strain by electroporation selecting for chloramphenicol resistance. The electroporation was done using a freezing step that significantly increased the frequency of electroporation. *B. anthracis* culture was grown O/N in 3 ml BHI 0.5% glycerol shaking at 37°C. 0.5 ml culture

was transferred to 50 ml BHI 0.5% glycerol (OD_{600}=0.1) in 500 ml E-flask. The sample was incubated at 200 rpm 37°C. (or 0.1-0.2 ml to 25 ml BHI 0.5% glycerol in 250 ml flask). At OD_{600}= 0.6-0.8 (approx 1 hour 45 min), bacteria were collected in 500 ml disposable sterile filter apparatus. The bacteria were washed 3 x 25 ml each with cold electroporation buffer (1 mM HEPES 10% glycerol pH 7.4). The cells were resuspended in 1/20 original volume (2.45 ml of

[0260] electroporation buffer for 50 ml culture) and kept on ice. The efficiency of electroporation can be enhanced by freezing the electrocompetent *B. anthracis* at –80°C. A 0.2 ml suspension of ice-cold or thawed electrocompetent *B. anthracis* cells were mixed with 1 micrograms (1 to 5 microliters of miniprep) of "very clean" unmethylated plasmid DNA to 0.2 ml cells suspension in a 0.2 cm gap electroporation cuvette (control=no DNA). The sample was then kept on ice for 15 min. The cells were then pulsed at 25 μFD, 200Ω, 2.5 kV (or, alternatively, 0.4 ml cells were pulsed in 0.4 cm cuvette at 400Ω). Time constant was approximately 4-5 msec. Immediately after pulse, 1 ml BGGM (BHI containing 10% glycerol, 0.4% glucose and 10 mM $MgCl_2$) was added. The cells are transfered to a sterile polypropylene tube and incubated 37°C 1 1/2 hour, shaking. The cells are pelleted, resuspended in 200 microliters BGGM and plated on selective media.

[0261] The pKSV7-*dl uvrAB* was integrated into the *B. anthracis* chromosome at 41° C. pKSV7-*dl uvrAB* was allowed to excise and cure at the permissive temperature, resulting in chloramphenicol sensitive colonies. PCR primers were designed to detect the deletion on the chromosome. 20% of the chloramphenicol sensitive colonies harbored the deletion in the B *anthracis* chromosome. PCR analysis of the *uvrAB*$^-$ strain indicated retention of the pXO1 virulence plasmid.

EXAMPLE 3

S-59/UVA treatment of *Bacillus anthracis* Sterne strain with and without *uvrAB* deletion.

[0262] Two *uvrAB*$^-$ clones constructed as indicated in Example 2 (clone 8 and clone 32A) were S-59-treated, along with the parent strain, by growing in BHI at 37 °C at 300 rpm to an OD_{600} of 0.3, at which point 50 mL of solution was transferred to a clean flask and S-59 was added to the concentrations indicated in Table 2. These samples were incubated at 37 °C at 300 rpm with vigorous shaking for approximately 1 hour (OD_{600} approximately 1.0, approximately 1

x 10^9/mL). A 1 mL aliquot was removed to assess the titer and the remaining was transferred to a 150 mm Petri dish and irradiated at a UVA dose of 6 J/cm^2 (FX-1019), resulting in a six-log reduction in titer, as compared to the parental strain, as indicated in Table 2, below, and Figure 1.

Table 2 Attenuation of *Bacillus anthracis* Sterne strain vs. *uvrAB*$^-$ mutant with psoralen S-59/UVA treatment.

S-59	Bacterial log titer			Log attenuation		
nM	Sterne	*uvrAB*$^-$ (1)	*uvrAB*$^-$ (2)	Sterne	*uvrAB*$^-$ (1)	*uvrAB*$^-$ (2)
0	8.26	8.13	8.31	-	-	-
25	-	7.46	7.45	-	0.67	0.86
50	-	6.31	6.28	-	1.82	2.03
100	-	3.11	3.68	-	5.02	4.63
200	6.84	<1	<1	1.42	>7.13	>7.31
400	-	<1	<1	-	>7.13	>7.31
500	5.29	-	-	2.97	-	-
1000	3.11	<1	<1	5.15	>7.13	>7.31
1500	1	-	-	>7.26	-	-
2500	1	-	-	>7.26	-	-
5000	1	-	-	>7.26	-	-

EXAMPLE 4

Asporogenic *B. anthracis* vaccine strains

[0263] *The spoIIE in-frame deletion.* In the following examples, the *spoIIE* gene mutant is a deletion mutant, while the *cya* gene mutation is a point mutation and the *lef* gene mutation is a point mutation.

[0264] The *spoIIE* region of *B. anthracis* is identified by homology to the same gene in *B. subtilis*. In order to isolate an in-frame deletion of *B. anthracis* SpoIIE, the *spoIIE* gene is first amplified by PCR and cloned it into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA). Next, most of the *spoIIE* gene is deleted by using the technique of gene splicing by overlap extension

(SOE) (Horton et al., Biotechniques 8:528-35 (1990)). This in-frame deleted *spoIIE* gene is cloned into the shuttle vector pKSV7, which carries a chloramphenicol-resistance gene and cannot replicate at 42°C (Smith et al., *Biochimie*, 74:705-11 (1992)). pKSV7 containing the deleted *spoIIE* gene is then electroporated into *B. anthracis*, and cells are grown at 42°C in the presence of chloramphenicol to select for strains in which the plasmid has integrated by homologous recombination into the *spoIIE* gene. Further growth at 30°C without chloramphenicol selection allows excision and loss of the plasmid. Chloramphenicol-sensitive strains should be found at about 1%, and about half of them should contain the deleted *spoIIE* allele (Camilli et al., (1993)). The presence of the deletion is confirmed by PCR and Southern blot analyses.

[0265] An in-frame deletion of the *spoIIE* gene of *B. anthracis* was generated by SOE PCR. Briefly, a fragment containing the 5' region of spoIIE and sequences upstream was amplified with primers Spo2A and Spo2B. Primer Spo2A was: TAACGACCGCGCTCCAAAAG (SEQ ID NO:30). Primer Spo2B was: GGCATATTTTTCTTCACTTTTGCCCACTTTACTCCTCCAAGCTGACC (SEQ ID NO:31). A fragment containing the 3' region of spoIIE and downstream sequences was amplified with primer spo2C and primer spo2D. Primer spo2C was: GGTCAGCTTGGAGGAGTAAAGTGGGCAAAAGTGAAGAAAAATATGCCG (SEQ ID NO:32). Primer spo2D was: TGCATTCATCCCGTATTGCT (SEQ ID NO:33). The fragments were combined and the overlapping region was annealed, yielding a product that was amplified with primers spo2A and spo2D. This fragment of the spoIIE region contains the putative promoter region of spoIIE and encodes an allele of spoIIE from which amino acids 12-787 are deleted. The construct was cloned into the topo-TA plasmid and the sequence was confirmed. This allele was subcloned into the tempterature sensitive shuttle vector pKSV7, and allelic exchange was performed. The presence of the deletion allele and absence of the wild-type allele was confirmed by PCR and by Southern blotting.

[0266] *The spoIIE/uvrAB double deletion strain.* Starting with the *spoIIE* deletion strain, an in-frame deletion of the *uvrA* and *uvrB* genes is made. Once again, the genes of interest are amplified and and cloned into pCR-Blunt II-TOPO. Then we shall delete most of the *uvrA* and *uvrB* genes by the SOE technique. This in-frame deleted *uvrAB* region is cloned into pKSV7, and the construct is electroporated into the *B. anthracis spoIIE* deletion strain.

Chloramphenicol-resistance is selected at 42°C in order to select for the integration of the plasmid into the *uvrAB* region. Growth at 30°C without drug selection is allowed in order to encourage the growth of segregants that have lost the plasmid. Chloramphenicol-sensitive colonies are picked and tested by PCR for loss of the *uvrAB* region, and that loss is confirmed by Southern blot analysis.

EXAMPLE 5

A temperature sensitive *recA* mutant of *B. anthracis*

[0267] To generate a temperature sensitive *recA* mutant of *B. anthracis* which grows well at 30°C and is very sensitive to psoralen at 42°C, a mutation is made in *B. anthracis* which is analogous to the V246M mutation of the temperature sensitive *recA* mutant of *E. coli*, recA44 (Kawashima et al. *Mol. Gen. Genet.* 193:288-92 (1984)). To make the *B. anthracis* mutant, the sequence 245KVVKNK250 (SEQ ID NO:14; numbering is according to Kawashima et al. (1984)), which is conserved between *E. coli* and *B. anthracis*, is mutated. The V246M mutation of recA44 (corresponding to a V244M mutation in *B. anthracis* RecA) is introduced into the cloned *B. anthracis recA* gene by mismatched oligonucleotide mutagenesis, using the Stratagene Quick Change kit (Stratagene, La Jolla, CA). The mutations are confirmed by sequence analysis, and the mutated gene is transferred into pKSV7, in order that they can be introduced into the chromosome of *B. anthracis spoIIE uvrAB* by allelic exchange. Alternatively, the *recA* gene from the *B. anthracis* strains is deleted and replaced with the recA44(ts) allele of *E. coli*. (It is known that *B. anthracis recA* functions in *E. coli* (Ko et al., J. Bacteriol 184:3917-22 (2002)).)

[0268] In the wild-type *E. coli* RecA coding sequence SEQ ID NO:50, below, (GenBank Acc. No. V00328), a mutation in the coding sequence that would encode a mutation analogous to that of *E. coli* recA44 would comprise a mutation of "g" to "a" at nucleotide 739. This would encode a RecA protein comprising a V to M mutation at amino acid 247. Amino acid Val-247 occurs in the context: 245-VKVVKNK-251 (SEQ ID NO:56). The position of the amino acid, indicated by Kawashima, et al., supra, to reside at amino acid-246 in *E. coli* recA, occurs in the RecA coding sequence of GenBank Acc. No. V00328 at amino acid-247 of *E. coli* recA. The protein sequence of the *E. coli* RecA of GenBank Acc. No. V00328 in which the temperature sensitive mutation has been made is shown in SEQ ID NO:52, below.

[0269] Valine-244 of the *B. anthracis* recA wild type sequence corresponds to the valine-247 of the *E. coli* recA wild type sequence of (GenBank Acc. No. V00328) and valine-246 of the sequence referred to in Kawashima, et al., supra, (the amino acid that is mutated to methionine-247 in the temperature sensitive *E. coli* mutant). Valine-244 of the *B. anthracis* recA wild type sequence occurs in the context: 242-VKVVKNK-248 (SEQ ID NO:56). Thus, mutating valine-244 of the *B. anthracis* recA wild type sequence to a residue encoding methionine-244 is expected to produce a temperature sensitive recA mutant. Such a protein sequence is shown as SEQ ID NO:54, below.

EXAMPLE 6

Introduction of mutations in the active sites of *B. anthracis* antigens

[0270] As mentioned above, in the following examples, the *spoIIE* gene mutant is a deletion mutant, while the *cya* gene mutation is a point mutation and the *lef* gene mutation is a point mutation. The lethal factor mutation H686A inactivates its protease activity, and the edema factor mutations K346Q and K353Q (together) inactivate its adenyl cyclase activity (Brossier et al., Infect. Immun., 68:1781-1786 (2000)). These mutations are introduced into *B. anthracis* strains to be used in vaccines, such as the *spoIIE uvrAB* and *spoIIE uvrAB recA*ts strains. The *lef* (lethal factor) and *cya* (edema factor, adenyl cyclase) genes are cloned and mutagenized with the Quick Change kit (Stratagene, La Jolla, CA) to create the mutant genes. The mutant genes are then transferred to pKSV7 and finally introduced into the host pXO1 plasmid by allelic exchange.

[0271] Mutagenesis of *Bacillus anthracis* toxin genes was accomplished as follows:

[0272] Generation of edema factor mutations K346Q and K353Q (together) that inactivate its adenyl cyclase activity used the following protocol. A fragment of cyaA gene was amplified with primers: cyaF: AGATAAAATACAGCAGACACAAGAC (SEQ ID NO:34)
cyaR: TAGTTGAATCCGGTTTCCTC (SEQ ID NO:35),

[0273] and cloned into the topo TA vector. To create the mutant allele primers were used in the Quick Change® kit (Stratagene, La Jolla, CA):
cyal1F: GGTGTGGCTACACAGGGATTGAATGTTCATG (SEQ ID NO:36)
cyal1R: CATGAACATTCAATCCCTGTGTAGCCACACC (SEQ ID NO:37)

[0274] Mutation of the second residue was effected using the following primers:

cyal2F: GATTGAATGTTCATGGACAGAGTTCGGATTGGG (SEQ ID NO:38)

cyal2R: CCCAATCCGAACTCTGTCCATGAACATTCAATC (SEQ ID NO:39)

[0275] The following primers introduced a silent mutation, which was used to check for mutagenesis:

cyaMSF: ACAGGGATTGAATGTCCATGGACAGAGTTCG (SEQ ID NO:40)

cyaMSR: CGAACTCTGTCCATGGACATTCAATCCCTGT (SEQ ID NO:41)

[0276] This allele was transferred to pKSV7 and then introduced into the host pXO1 plasmid by allelic exchange. The mutation was verified by PCR followed by a restriction digest that discerns between the wild type and mutant alleles.

[0277] Generation of a mutation in lethal factor H686A that inactivates its protease activity, was accomplished as follows. A fragment of the *lefA* gene (encoding lethal factor) was amplified with primers CAGGAGGGTTAATTGATAGTCCGTC (SEQ ID NO:42) and TTTGCGTCTTATTTGGCTTTAACG (SEQ ID NO:43), and the fragment was cloned into the topo-TA vector (Invitrogen). The following primers were then used to mutate the residue encoding H686 to A using the Quick Change® kit (Stratagene, La Jolla, CA): LefMF: AGTGAGGGTTTTATAGCTGAGTTTGGACATGCTGTG (SEQ ID NO:44) and LefMR: CACAGCATGTCCAAACTCAGCTATAAAACCCTCACT (SEQ ID NO:45). This allele has been transferred to pKSV7. Once transferred to pKSV7, the allele is introduced into the host pXO1 plasmid by allelic exchange.

EXAMPLE 7

Inducible expression of protective antigen at high levels

[0278] *The use of SOS regulatory sequences for expressing protective antigen at high levels.* Cheo et al (Cheo et al., *J. Bacteriol.*, 175:5907-15 (1993)) have shown that the consensus sequence $GAACN_4GTTC$ (SEQ ID NO:15) defines the LexA repressor site for genes in the SOS response of *B. subtilis*. This sequence was identified within the promoter regions of DNA damage-inducible (din) genes from *Bacillus subtilis*. This sequence has been proposed to function as an operator site that is required for regulation of the SOS system of *B. subtilis*, and the consensus sequence was modified to 5'-CGAACRNRYGTTYC-3' (R=G or A; Y = C or T;

N = A, G, C, or T; SEQ ID NO:29) by Winterling et al (Winterling et al., *J. Bacteriol.*, 180:2201-221 (1998)). A similar consensus sequence upstream of the promoters for the *B. anthracis recA* and *uvrAB* genes, which are part of the SOS regulon, is identified. To make a *B. anthracis* strain in which expression of protective antigen is induced in response to photochemical treatment, the protective antigen gene is put under the control of the SOS regulatory sequence and introduced into the *B. anthracis spoIIE ΔuvrAB* strain, so that treatment with psoralen and UVA light will induce expression of high levels of protective antigen to be made. The gene of interest, in this case, the protective antigen gene, is functionally linked with a *B. anthracis* promoter that is under the control of the SOS response.

[0279] Homologous recombination using the pKSV7 vector as described above in Example 2 is used to insert the heterologous cassette expressing protective antigen at a desired site in the chromosome. This can include replacement of the homologous protective antigen with the modified heterologous sequence.

[0280] *The use of other inducible promoters.* In some embodiments, for instance, when the *B. anthracis* strain carries both *uvrAB* and *recA* mutations, the SOS response will not occur, since this response depends upon RecA protein. In these cases, it is desirable to use a different sort of inducible promoter. Suitable promoters for this use can be determined by first identifying which proteins are expressed at high levels after S-59/UVA treatment of an *uvrAB recA* double mutant. The mass spectrometry techniqued described in Lenz et al., *Proc. Natl. Acad. Sci. USA*, 100:12432-12437 (2003), can, for instance, be used for this purpose. Once the proteins expressed at high level under S-59 UVA treatment conditions are determined, the promoters controlling expression of the highly expressed proteins can be identified through techniques known to those of ordinary skill in the art. A promoter identified in this manner can then be fused to the gene expressing the protective antigen. The construct can then be introduced into the chromosome of the mutant *Bacillus anthracis* using one of the integration vectors described herein or another vector known in the art. The present invention provides a heterologous promoter operably linked with a nucleic acid encoding, e.g., protective antigen.

EXAMPLE 8

Exemplary mutant *B. anthracis* strains

[0281] A variety of different mutant *B. anthracis* strains are prepared using combinations of the methods described in the Examples, above. Exemplary mutant *B. anthracis* strains to be used in vaccine compositions are listed in Table 3.

Table 3. *B. anthracis* strains and candidate vaccines

Strain and/or Genotype	Relevant Characteristics and Phenotype	Use and Vaccine Strain Number
Ames pXO1+/pXO2+	Fully virulent wild-type *B. anthracis* (Toxigenic and encapsulated)	Initial host strain for construction of all vaccine candidates Production of virulent spores for challenge experiments in mice and guinea pigs
Sterne pXO1+/pXO2-	Toxigenic, non-encapsulated	Production of virulent spores for challenge experiments in mice and guinea pigs
spoIIE pXO1+/pXO2+	Non-sporogenic Toxigenic, encapsulated	Vaccine strain #1
SpoIIE/uvrAB pXO1+/pXO2+	Non-sporogenic NER-[1] (Increased S-59/UVA sensitivity) Toxigenic, encapsulated	Vaccine strain #2
SpoIIE/uvrAB/recA ts[3] pXO1+/pXO2+	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Toxigenic, encapsulated	Vaccine strain #3
SpoIIE/uvrAB/recA ts pXO1+/pXO2+	Non-sporogenic NER-/conditional HR-[4] (Increased S-59 /UVA sensitivity) Toxigenic, encapsulated	Vaccine strain #4
spoIIE/uvrAB/ pXO1 (*lef*686/*cya*346/35)/ pXO2+	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Encapsulated Non-toxigenic (LF/EF functional domains mutated)	Vaccine strain #5
spoIIE/uvrAB/ recA ts/ pXO1(*lef*686/*cya*346/35)/ pXO2+	Non-sporogenic NER-/conditional HR- (Increased S-59/UVA sensitivity) Encapsulated Non-toxigenic (LF/EF functional domains mutated)	Vaccine strain #6

spoIIE/uvrAB/ pXO1 (*lef*686/*cya*346/35)/ pXO2+/ Pro_{S-59}-PA	Non-sporogenic NER- (Increased S-59/UVA sensitivity) Encapsulated Non-toxigenic (LF/EF functional domains mutated) S-59 psoralen inducible PA	Vaccine strain #7
spoIIE/uvrAB/ recA ts/ pXO1(*lef*686/*cya*346/35)/ pXO2+/ Pro_{S-59}-PA	Non-sporogenic NER-/conditional HR- (Increased S-59/UVA sensitivity) Encapsulated Non-toxigenic (LF/EF functional domains mutated) S-59 psoralen inducible PA	Vaccine strain #8

[1]NER, nucleotide excision repair
[3]Conditional *recA* strains under the control of a *lacI* repressible promoter can also be derived
[4]HR, homologous recombination

EXAMPLE 9

Characterization of protein expression levels, including protective antigen and capsule, in psoralen-inactivated *B. anthracis* strains

[0282] To show that inactivated *B. anthracis* strains can still metabolize, the cells are incubated in minimal medium with bicarbonate (Thorne et al., J. Gen. Microbiol.,17:505-516 (1957)). After such incubation the cells are removed by centrifugation and save the supernatant. The supernatant is subjected to SDS-polyacrylamide gel electrophoresis. After staining with Coomassie Blue, protective antigen stands out, and its presence is confirmed by Western blot analysis (Brossier et al., Infect. Immun. 68:5731-5734 (2000)) and by mass spectometry. In addition, mass spectrometry is used to identify the other proteins that are excreted under these conditions, using the methods described in Lenz et al., Proc. Natl. Acad. Sci. U.S.A., 100:12432-12437 (2003). In order to assess whether polyglutamate capsule is made under these conditions, pXO2, which encodes the genes for capsule synthesis, is introduced into the strains by transduction and (Green et al., Infect. Immun., 49:291-297 (1985). Capsule is measured by rocket immunoelectrophoresis (Uchida et al., Mol. Microbiol, 23:1229-1240 (1997)).

EXAMPLE 10

Characterization of the humoral and mucosal responses in Swiss Webster and A/J mice immunized with attenuated *B. anthracis* strains

[0283] *Mouse Immunization.* Mice are injected with the S-59/UVA vaccines by the intramuscular (IM) or the subcutaneous (SC) routes to determine which route of immunization results in the best bacterial-specific humoral and cellular responses. Intranasal (IN) immunization of mice is also tested to assess mucosal responses induced by the candidate vaccines. IN immunization with 5 μl of a designated vaccine preparation into each nare of lightly anesthetized mice is performed as described previously (Boyaka, et al., *J. Immunol.*, 170: 5636-5643 (2003)). Mice are immunized with 0.1 LD_{50} doses of the candidate vaccines. Any of the eight S-59/UVA inactivated vaccine candidates in which a median lethality level is not observed is given at an initial dose of 10^8 particles. Mice that are immunized by more than one route are not injected with a combined dose that exceeds the 0.1 LD_{50} dose, or is greater than 10^8 particles. Mice given multiple immunizations receive consistent vaccine doses with all injections. As immunization on three consecutive days with S-59/UVA inactivated *Listeria uvrAB* resulted in increased humoral and cellular immunity as compared to a single immunization, the same strategy is used with the *B. anthracis* strain vaccines. Mice are also given booster immunizations at 14 days and 28 days following the primary immunization.

[0284] *Quantification of antibodies to PA, LF, EF, capsule, and whole bacteria.* The mucosal and antibody responses in mice immunized with the various vaccine candidates are characterized. Serum is taken from the retroorbital plexus prior to immunization as well as 1 week after each immunization. Saliva and nasal washes for measurement of IgA levels are performed at the time of sacrifice one week after the final immunization. The durability of the humoral and mucosal immunity induced by the candidate vaccines at 45 days after the final immunization is also characterized . Humoral and mucosal responses against PA, capsule, and vegetative bacteria (Sterne strain) are determined by enzyme-linked immunosorbent assays (ELISAs), as published previously (Ballard et al., *Proc. Natl. Acad. Sci. U.S.A,* 93:12531-12534 (1996); Rhie et al., *Proc. Natl. Acad. Sci. U.S.A.*, 100:10925-10930 (2003)). Briefly, Immulon 96-well Maxisorp plates (Nalge Nunc) are first coated by 5μg purified PA, LF, EF, BSA conjugated with poly-γ-D-glutamic acid (PGA) capsule prepared as described previously (Rhie

et al., *Proc. Natl. Acad. Sci. U.S.A*, 100:10925-10930 (2003)), or with S-59 psoralen/UVA inactivated bacteria ground under liquid nitrogen using a mortar and pestle in 50mM carbonate buffer (pH 9.6) at 4°C for 16 h, and blocked with TSTA buffer (50mMTris (pH 7.6), 142 mM NaCl, 0.05% sodium azide, 0.05% Tween 20, 2% bovine serum albumin). Serial two-fold dilutions of mouse plasma or mucosal secretions are added to the 96-well plates coated with PA, PGA-BSA, or Sterne respectively. Binding of Abs to the immobilized antigens is determined by incubation with isotype-specific peroxidase goat anti-mouse μ, γ, or α H chain-specific antibodies from Southern Biotechnology Associates (Birmingham, AL). Biotinylated rat anti-mouse γ 1 (clone G1-7.3), γ 2a (clone R19-15), γ 2b (clone R12-3), or γ 3 (clone R40-82) H chain-specific mAbs (BD PharMingen, San Diego, CA) and streptavidin-conjugated peroxidase are used for IgG Ab subclass analysis (Cole, *J. Bacteriol.*, 107:846-852 (1971); Cole et al., *Basic Life Sci.*, 5B:487-495 (1975)). The colorimetric reaction is developed by addition of ABTS substrate (Sigma-Aldrich, St. Louis, MO). End-point titers are expressed as the reciprocal $\log_2$ dilution giving OD_{415} greater than two standard deviations above those obtained with control, non-immunized mice.

[0285] *Enzyme-linked immunospot (ELISPOT) assay for the detection of Ig-secreting cells.* The frequency of PA-specific Ig-secreting lymphocytes is determined by ELISPOT analysis (Boyaka et al., *J. Immunol.*, 170:5636-5643 (2003)). Briefly, spleens or cervical lymph nodes of vaccinated and control mice are rapidly dissected out and placed in ice-cooled RPMI 1640 medium and single cell suspensions are prepared. 96-well PVDF-based plates (BD Biosciences, San Jose) are coated overnight with 2.5 μg/ml purified PA (List Biological Laboratories, Campbell, CA). The plates are washed, blocked for 2 hrs at 37°C with 200 μl complete RPMI, and serial dilutions of cell suspensions are added to 96-well plates. Cells are incubated on the plates for 6 hours at 37°C in 5% CO_2. Antigen-specific Antibody Forming Cells (AFC) are detected with isotype-specific biotin-labeled anti-mouse μ, γ, or α H chain-specific antibodies (Southern Biotechnology Associates). After incubation at RT for 2 h, the plates are washed, and goat anti-biotin:1nm Gold conjugate (GAB1; Ted Pella) is added for 1 hour at RT. After extensive washing, 30 μl of the silver substrate (Silver Enhancing Kit; Ted Pella) is added into each well and the spot development is monitored. Spots in each well are counted using an automated ELISPOT plate reader (CTL, Cleveland). The humoral response is expressed as the number of antibody forming cells per 106 spleen or lymph node cells.

[0286] *Toxin Neutralization Assays.* Neutralizing antibodies induced in mice immunized with the vaccine candidates are evaluated for the ability to protect the J774 macrophage cell line from lethal toxin (PA+LF) (Mock et al., Annu. Rev. Microbiol., 55:647-671 (2001); Boyaka et al. (2003); Rhie et al., *Proc. Natl. Acad. Sci. U.S.A*, 100:10925-10930 (2003)). Briefly, J774 cells (ATCC, Manassus, VA) are added to 96-well flat-bottom plates (Nunc) at 5 $X10^4$ cells/well and incubated for 12 hours at 37° C in 5% CO_2. Test serum or mucosal secretions are serially diluted two-fold in TSTA buffer. PA and LF (400 ng/ml PA and 40 ng/ml LF) are added to the antiserum dilutions. After incubation for 1 hour the antiserum/lethal toxin complex mixture is added to the cell suspension and incubated for an additional 5 hours. Cell viability is monitored by the MTT assay (absorbance measured at 540 nm). Assays are performed in triplicate with a negative control (normal serum) and a positive control (MAbs, 14B7 and 1G3) (Mikesell et al., *Infect Immun.*, 39:371-376 (1983); Starnbach et al., *Nature Medicine*, 9:996-997 (2003)) included in each plate. The mean and standard deviation of each triplicate sample dilution is calculated. The endpoint is expressed as the highest serum dilution exhibiting 50% neutralization of the anthrax toxin as compared to normal control serum.

EXAMPLE 11

Characterization of the PA-, LF-, and EF-specific CD4+ T cell-mediated responses in A/J mice vaccinated with modified *B. anthracis*

[0287] *T cell Proliferation.* CD4+ T cell proliferation are determined from PBMC, spleen and lymph node cells of vaccinated and naïve A/J mice. Spleen and cervical lymph nodes are dispersed to obtain single cell suspensions as previously described (Boyaka et al., *J. Immunol.*, 162: 122-8 (1999); Lillard et al., *J. Immunol.*, 166:162-169 (2001); Little et al., *Infect. Immun.*, 65:5171-5 (1997)). CD4+ T cells are isolated by negative selection using the Mouse CD4+ T cell isolation kit from Miltenyi Biotec (Auburn, CA). Purified CD4+ T cells from individual mouse spleens, from pooled lymph nodes or PBMCs are cultured at 4 x 10^6 cells/ml and stimulated with varying concentrations of PA, LF or EF in the presence of T-cell-depleted, non-dividing syngeneic naïve spleen feeder cells (8 X 10^6 cells/ml) in complete RPMI (RPMI supplemented with 10% FBS, 10mM Hepes, 2 mM L-glutamine, 1mM sodium pyruvate, non-essential amino acids, 23.8 mM sodium bicarbonate, 5 X 10^{-5} M μ-Mercapthoethanol, 100 U/ml

penicillin and 100 μg/ml streptomycin). The replication of splenic feeder cells is arrested by brief photochemical treatment with S-59 psoralen. Cultures are incubated for 4 days at 37°C and 5% CO_2 prior to addition of 0.5 μCi of tritiated thymidine ([^{3}H]TdR) for the final 18 to 20 hours. The cells are harvested onto glass fiber sheets and the amount of incorporated thymidine is determined by measuring the radioactivity on the scintillation counter (Wallac, Turku, Finland).

[0288] *Analysis of PA-, EF- or LF-induced cytokine responses.* CD4+ T cells are isolated by negative selection using the Mouse CD4+ T cell isolation kit from Miltenyi Biotec (Auburn, CA). Purified CD4+ T cells from spleens or lymph node of individual mice are cultured in round-bottom 96-well plates at 1 X 10^5 cells/well and stimulated with varying concentrations of PA, LF or EF in the presence of T cell-depleted, non-dividing syngeneic naïve spleen feeder cells (1 X 10^5 cells/well) in complete RPMI. The T cell-depleted spleen feeder cells are arrested by a brief photochemical treatment with S-59. T cell cultures are incubated for 2 days at 37°C and 5% CO_2. Expression of T helper-1 and T helper-2 cytokines is determined from supernatants of antigen-stimulated CD4+ T cells using the Th1/Th2 Cytometric Bead Array kit (BD Pharmingen, San Diego, CA).

EXAMPLE 12

Characterization of the extent of protection against spore and lethal toxin challenge in Swiss Webster and A/J mice at 45 days post last immunization dose with modified *B. anthracis* vaccines

[0289] *Protection of mice against lethal toxin challenge.* Mice immunized with selected candidate vaccines are challenged by tail vein injection with lethal toxin, as described previously (Price et al., *Infect. Immun.*, 69:4509-15 (2001); Rhie et al., *Proc. Natl. Acad. Sci. U.S.A.*, 100:10925-10930 (2003)). Lethal toxin is prepared by mixing recombinant PA and LF recombinant proteins (List Biological Laboratories, Campbell, CA) as described (Rhie et al. (2003)). The lethal toxin IV LD_{50} per mouse is approximately 12 μg of PA mixed with 6 μg of LF. The median lethality in mice of freshly prepared lethal toxin is determined by tail vein injection over a 0.1-10 LD_{50} dose range of the published values. The protection studies include lethal toxin challenge over a range of 5-10 times the LD_{50} dose. In this model, unprotected mice succumb within 24 h. Initially, death by anthrax is confirmed in selected mice by plating blood

on tryptic soy agar and incubating overnight at 37° C. Plates are observed for colonies with 2-3mm typical *anthracis*-like "ground glass" appearance. All mice treated with lethal toxin are monitored daily, and experiments are terminated after 2 weeks and all protected mice are sacrificed.

[0290] *Spore preparation.* Sterne strain spores are prepared as described (Barnard and Friedlander, 1999). Briefly, single colonies are inoculated into 5 ml of FA medium (3.3% tryptone, 2% yeast extract (dialyzed overnight against water), 0.2% L-histidine, 0.8% Na_2HPO_4, 0.4% KH_2PO_4, 0.74% NaCl) contained in a 100-ml bottle and shaken for 5 h at 37°C. One-tenth-milliliter aliquots are spread on L agar plates, and incubated at 37°C. Bacterial lawns are scraped from the plates, washed extensively with sterile water, heat shocked for 30 min at 60°C, washed with water, purified on 58% Renografin-76 (Bristol-Myers Squibb, Princeton, N.J.) in water, as previously described (Palucka et al., *Nature Medicine*, 5:868-870 (1999)), and washed once more with water. The spores are then sedimented to a pellet at 10,000 x g and resuspended in 1% phenol in water. This yield of this process has been published to range from 0.5 x 10^9 to 5.0 x 10^9 spores per plate.

[0291] *Protection of mice against lethal spore challenge.* The LD_{50} value of heat-shocked Sterne strain spores given by intramuscular (IM) injection is determined over a dose range of 10^3 to 10^8 spores. To evaluate protection in vaccinated mice against inhalation anthrax, challenge experiments are also performed by intratrachial (IT) spore administration, as described previously (Brook et al., *J. Med. Microbiol.*, 50:702-11 (2001)). Briefly, the tongue of immobilized and anesthetized mice are gently pulled outward and laterally with forceps, and the vaccine is delivered using a syringe fitted with a blunt 1.5 inch 22-gauge needle bent at a gentle angle, approximately 1 inch from the tip. We anticipate that the Sterne strain LD_{50} value administered by IM or IT routes is approximately 10^3 in A/J mice, and up to 10-fold higher in Swiss Webster mice. The protection studies include up to 100 LD_{50} dose spore challenge. All mice treated with spores are monitored daily, and experiments are terminated after 2 weeks and all protected mice are sacrificed. In all challenge experiments, the mean time to death is determined in non-surviving cohorts. In additional challenge experiments, the protection of mice against a fully virulent Ames strains (pXO1+, pXO2+) is tested.

EXAMPLE 13

Sequences useful in production of recombinant or mutant *Bacillus anthracis* strains

[0292] Recombinant and/or mutant *Bacillus anthracis* strains are described in the Examples above. Information is provided in the Table 4, below regarding some sequences of use in construction of some of the recombinant *B. anthracis* strains described above. Each of the sequences identified by accession number or by other reference in Table 4 is incorporated by reference herein in its entirety.

Table 4. Additional *B. anthracis* sequence information (including Genbank accession numbers)

Bacteria	Accession #	Gene	Location	Coordinates in the genome or plasmid
B. anthracis Ames	NC_003997	*spoIIE*	Chromosome	64936-67314
B. anthracis Sterne	NC_001496	*cya* (encodes edema factor)	pXO1 virulence plasmid	154224-156626
B. anthracis Ames	NC_007322 .	*pagA* (encodes protective antigen)	pXO1 virulence plasmid	143779-146073
B. anthracis Ames	NC_007322	*lef* (encodes Lethal Factor)	pXO1 virulence plasmid	149357-151786
B. anthracis Ames	NC_003997	*lexA* (lex repressor)	Chromosome	3453806-3454426
B. anthracis Ames	NC_003997	*recA*	Chromosome	3590268-3591626 (Intron 3590691-3591017; Ko M., et. al., J Bacteriol. 2002. 184:3917-3922)
B. anthracis Ames	NC_007530	*uvrC*	Chromosome	4324547-4326331

[0293] In addition, the *B. subtilis* SOS promoter binding region is identified as 5'-CGAACRNRYGTTYC-3' (R=G or A; Y = C or T; N = A, G, C, or T; SEQ ID NO:29) (Winterling, K.W. et. al., J Bacteriol. 1998 180:2201-2211).

[0294] The protein and nucleotide sequences corresponding to the genes listed in Table 4, above, are also provided below.

EXAMPLE 14

Psoralen-induced and heat-induced elimination of proliferative ability *B. anthracis* mutants

[0295] Figure 2 shows inactivation of various *B. anthracis* strains versus concentration of psoralen (S-59). Inactivation was measured by colony forming units (CFU). The S-59 concentrations were 0-2000 nM. The indicated *B. anthracis* strains were exposed to the indicated concentration of S-59, then treated with ultraviolet light (6.5 J/cm^2), followed by dilution and plating. During treatment with the psoralen and UV-light, the concentration of the suspended bacteria was an optical density of about OD = 0.6. The exposure time to the UV light was limited to about one minute. The *B. anthracis* strains were Sterne (-◇-)(open diamond); new Sterne (-□-)(open large square); SpoIIE (-△-)(open triangle); uvrAB (-▫-)(open small square); and uvrAB/SpoIIE (-O-) (open circle) (Figure 2). The results demonstrated that relatively low concentrations of psoralen, e.g., 100 nM or under 100 nM S-59, were sufficient for effecting nearly a 100 million-fold reduction in viable bacteria, where the *B. anthracis* was the uvrAB mutant or uvrAB/spoIIE mutant. The efficiency in reducing CFU was somewhat greater with the uvrAB/spoIIE mutant than with the uvrAB mutant. In contrast, much greater concentrations of S-59 were needed to effect similar reductions in CFU with the *B. anthracis* strains that were not uvrAB or uvrAB/spoIIE mutants. For example, the higher concentration of 1000 to 1200 nM S-59 were needed to produce a 10 million–fold reduction in viable bacteria, where the *B. anthracis* was Sterne, new Sterne, or the spoIIE mutant. Thus, the excision repair mutants of the present invention enable inactivation of *B. anthracis* at very low levels of psoralen (Figure 2).

[0296] Figure 3 shows colony forming units of *B. anthracis* before and after heating. The *B. anthracis* had been grown under sporulation conditions. The sporulation conditions used was PA medium. Heating was for 30 minutes at 68 degrees in aqueous solution. The following strains of *B. anthracis* were tested: Sterne; uvrAB mutant; spoIIE mutant, spoIIE/uvrAB/cyaA mutant, and SpoIIE/uvrAB mutant. Heat-treatment had no detectable influence on colony forming units of the Sterne strain or uvrAB mutant strain. These two strains did not contain

mutations in a spore-forming gene. But heat-treatment of the *B. anthracis* strains that were spoIIE mutant resulted a reduction of CFU by over 10-million fold, or in the range of 100-million fold. In short, the spoIIE mutation prevented spore formation by the heat-treated bacteria.

[0297] Many modifications and variations of this invention, as will be apparent to one of ordinary skill in the art, can be made to adapt to a particular situation, material, composition of matter, process, process step or steps, to preserve the objective, spirit, and scope of the invention. All such modifications are intended to be within the scope of the claims appended hereto without departing from the spirit and scope of the invention. The specific embodiments described herein are offered by way of example only, and the invention is to be limited by the terms of the appended claims, along with the full scope of the equivalents to which such claims are entitled; and the invention is not to be limited by the specific embodiments that have been presented herein by way of example. All publications, patents, patent applications, and accession numbers (including both polynucleotide and polypeptide sequences) cited herein are hereby incorporated by reference herein in their entirety for all purposes to the same extent as if each individual publication, patent, patent application or accession number were specifically and individually indicated to be so incorporated by reference.

ADDITIONAL SEQUENCES

[0298] Coding sequence of the *uvrA* gene of *Bacillus anthracis* (SEQ ID NO:1):

ATGGATAAGCCGGATGTAGATACGATTGAAGGCTTATCTCCAGCGATTTCAATCGAT
CAAAAAACGACGAGTCGTAATCCGCGTTCAACTGTTGGAACGGTAACGGAGATTTA
TGATTACTTACGTTTATTATTTGCGCGAATTGGTACGCCAATTTGTCCGAATCATGGC
ATTGAAATTACATCGCAAACAGTAGAGCAAATGGTAGACCGTGTACTTGAGTACCCT
GAACGTACGAAATTACAAGTGTTAGCTCCTATCGTTTCTGGGCGTAAAGGTGCACAT
GTAAAAGTACTTGAAGATATTAAGAAGCAAGGTTATGTTCGTGTACGTGTTGATGGT
GAAATGCTCGATGTGTCTGAAGATATTGCGTTAGATAAAAATAAGAAGCATTCTATT
GAAGTTGTAATTGACCGTATTGTTGTAAAAGAAGGAATCGCAAGCCGTCTTGCTGAT
TCTCTTGAAAGTGCATTAAAGCTTGGCGGGGGACGAGTGTTAATCGATGTTATGGGA
GAAGAGGAGCTTCTATTTAGTGAACATCATGCTTGTCCGCATTGTGGTTTTTCAATTG

GAGAATTAGAGCCGCGTATGTTCTCATTCAATAGTCCGTTCGGTGCATGTCCTTCTTG
TGATGGGCTTGGCTCAAAGTTAGAGGTAGATTTAGAACTTGTTAATTCCGAACTGGGA
TTTATCATTAAATGAGCATGCGATTGCGCCTTGGGAACCGACAAGTTCACAATATTA
CCCACAGCTTTTACAATCTGTATGTAATCATTATGGCGTGGATAATGGATGTGCCTGT
AAAAGATATACCGAAAGATTTATTTGATAAAGTGTTGTACGGAAGCGGTGAAGAGA
AAGTTTATTTCCGCTATGTAAATGAATTTGGTCAAGTAAAGGAAAATGAGATTTTAT
TTGAAGGTGTTATTCCAAATATTGAACGTCGTTATCGTGAGACGAGTTCGGATTACA
TTCGTGAGCAAATGGAAAAGTATATGGCAGAACAAGCTTGTCCGAAGTGTAAAGGC
GGACGCTTAAAGCCTGAAAGTTTAGCTGTTTTCGTTGGCGGAAAAACGATTGCTGAT
GTAACGAAGTATTCTGTTCAAGAAGTACAGGAATTCTTCTCAAATGTGGAGCTAACA
GAGAAACAACAAAAAATTGCCCATTTAATTTTAAGAGAAATTCAAGAGCGCGTTGG
GTTCTTAGTAAACGTTGGTTTAGATTATTTAACGTTAAGTCGTGCCGCAGGAACTTTA
TCTGGTGGTGAGGCGCAACGTATTCGTTTAGCAACGCAAATTGGTTCGCGTCTTACT
GGGGTGCTTTACATTCTTGATGAGCCTTCTATCGGTTTGCATCAGCGCGATAACGAT
CGTCTTATTCGTACATTGCAAGAAATGCGTGATTTAGGTAATACGTTAATTGTTGTTG
AGCATGATGAAGATACGATGATGGCAGCTGATTATTTACTGGATATCGGGCCTGGCG
CAGGTATTCACGGTGGACAAGTTGTATCAGCGGGTACACCAGCTGAAGTGATGCAA
GATGAGAATTCACTAACAGGTAAGTATTTAAGCGGTAAAGAGTTTATTCCAGTTCCA
CTTGAAAGACGTAAAGGTGATGGACGTAAAGTGGAGATTGTCGGTGCAAAAGAGAA
TAACTTAAAGAACGCGAAGATGTCATTCCCGCTTGGTACGTTTGTAGCGGTAACGGG
TGTATCTGGATCAGGTAAAAGTACGATGATTAATGAAGTACTATATAAATCGTTAGC
GCAAAAGTTATATAAAGCGAAAGCGAAGCCAGGTACTCATAAAGAAATTAAAGGTC
TTGAGCATTTAGATAAAGTTATCGATATTGATCAATCGCCAATCGGTCGTACACCAC
GTTCTAATCCAGCAACCTATACAGGTGTGTTCGATGATATTCGTGATGTGTTTGCGC
AAACGAATGAAGCGAAAGTGCGCGGATATCAAAAAGGGCGTTTCAGCTTTAACGTA
AAAGGTGGACGTTGTGAAGCGTGCCGTGGTGATGGAATTATTAAAATTGAGATGCA
CTTCTTACCAGACGTATACGTTCCGTGTGAAGTTTGTCACGGTAAACGTTACAACCG
TGAAACGTTAGAAGTGAAATATAAAGATAAAAACATTTCTGAAGTGTTAGGGATGA
CGATTGAAGACGGAGTAGAGTTCTTCGCTAATATCCCAAAAATTAAACGTAAACTTC
AAACGCTTGTAGACGTTGGGCTTGGTTATATGAAATTAGGGCAACCAGCCACGACTT
TATCTGGTGGTGAAGCACAGCGTGTGAAATTAGCTTCTGAATTACACCGTCGTTCTA

CAGGACGTACACTATACATTTTAGACGAGCCAACGACTGGTTTACATGCGCATGATA
TCGCCCGTCTTCTAGAAGTGCTGCAACGTCTTGTTGAGAGCGGTGAGACGGTACTTG
TCATTGAACATAATTTAGATGTAATTAAAACAGCGGATTATATCGTTGACCTTGGAC
CAGAAGGCGGAGACAAAGGTGGACAAATCGTTGCTTCCGGAACGCCAGAGCAAGT

[0299] Coding sequence of the *uvrB* gene of *Bacillus anthracis* (SEQ ID NO:2):

ATGGCTCACAATAAAACGTTAGCAGGACAGTTATATAGTGAGTTGAAAGACTTTTTC
CCGAATAATGCAGTTGAATATTTTGTTAGTTATTACGATTATTATCAGCCAGAAGCG
TATGTGCCACAAACAGATACGTTTATTGAAAAAGACGCGCAGATTAATGATGAAAT
CGATAAATTGCGTCACTCAGCAACGTCCGCATTATTTGAACGGGATGATGTAATTAT
TGTTGCGAGTGTTTCGTGTATATATGGTTTAGGTTCTCCAGAAGAATACCGCGAGTT
AGTTGTTTCACTTCGAGTTGGTATGGAAAAGGACCGCAATCAATTGCTTCGTGAACT
TGTTGATGTGCAGTATGGACGTAATGATATTGATTTCAAGCGTGGTACATTCCGCGT
GCGCGGAGATGTAGTTGAAATCTTCCCGGCATCACTTGACGAGCATTGCATTCGAAT
TGAGTTTTTTGGCGATGAAATTGATCGTATTCGCGAAGTAAATGCTTTAACGGGAGA
AGTATTAGCAGAACGTGATCATGTAGCAATCTTCCCAGCATCTCACTTCGTTACACG
TGAAGAAAAGATGAAGGTCGCTATTGAAAATATCGAAAAAGAATTAGAAGAGCGTT
TAAAGGAATTAAATGATAACGGTAAGTTGTTAGAAGCGCAGCGTATAGAACAGCGT
ACACGTTATGATTTAGAAATGATGCGCGAGATGGGCTTTTGTTCAGGGATTGAAAAC
TATTCCCGTCATTTAACACTTCGTCCAGCGGGTGCAACGCCGTATACGTTATTAGACT
ATTTCCCGAAAGATTTCTTAATCGTTATGGATGAGTCCCACGTATCAGTGCCGCAAG
TAAGAGCGATGTATAACGGGGACCAAGCGCGTAAACAAGTGCTTGTGGATCATGGA
TTCCGTCTGCCATCAGCTTTAGATAATAGACCGCTCACATTTGATGAGTTTGAAGAG
AAAACGAATCAAGTTATTTACGTTTCAGCAACGCCAGGACCGTATGAATTAGAGCA
GTCGCCAGAAGTAATAGAACAAATTATTCGTCCAACAGGGCTTTTAGATCCGCCAAT
TGATATACGACCAATTGAAGGGCAGATTGACGATCTATTAGGAGAGATTCAAGATC
GCATTGCAAAAAATGAACGTGTATTAATTACAACTTTAACGAAGAAGATGTCAGAG
GATTTAACAGACTACTTAAAAGATGTAGGAATTAAGGTGAATTATCTGCATTCTGAA
GTGAAAACGTTAGAACGTATTGAAATTATACGAGATCTTCGCCTTGGTAAGTTTGAT
GTTCTCGTTGGTATTAACTTATTGCGAGAAGGATTAGATATTCCAGAAGTATCCCTT
GTAGCTATTTTAGATGCCGATAAGGAAGGATTCTTGCGTTCAGAGCGTTCGTTAATT
CAAACAATTGGCCGTGCAGCACGTAATGAAAACGGTCGCGTTATTATGTACGCAGA

TCGTATAACGAGATCGATGGGGATTGCGATTGAAGAGACGAAGCGTCGTCGTAGTA
TACAAGAAGCTTACAATGAAGAGCATGGTATTACGCCGAAAACGATTCAAAAAGGT
GTGCGTGATGTAATCCGTGCAACGACAGCTGCTGAAGAGCCGGAAACATATGAAGC
GACGCCAGCTAAGAAGATGACGAAAAAAGAGCGTGAAAAGACAATTGCGAAGATG
GAAGCAGAAATGAAAGAAGCAGCAAAAGCA

[0300] Protein sequence of Stage II Sporulation Protein E (SpoIIE) from *Bacillus anthracis* (SEQ ID NO:16):

[0301] MKFEQVFFRWGFIIVVIGFLLGRAYILTNILPFALPFFAAVYVMKRDKMPL
AFLALMGGALSVSIDNLFFTFASIFTFFIYNIFFSRFTRKTVGLVPFQVFISALTAHLVVVY
FAQQTVTMYDLLVSTIEAGLSFVLTMIFLQSVPLLVERKGKQQALETEEIVCLIILLASVL
TGTTDWFVYDASIQHIFTRYLVLVFAFIAGAATGSTVGVVTGLILSLANVSSLSQLSLLAF
SGLLGGLLKEGKRIGVSLGLLIGTSLITLYVDKQTNIVTTLIESGVAIAFFLLTPKLVMDRI
AKFMPGTQEHSQDQQQYLRRMRDVTANKINQFANVFAALSNSFSVYGYVEEEDKETEA
DLFLSTITAKTCQTCFKKDQCWVVNFDKTYDYMKQIMSETEEGTLQHNRKLVREWDK
HCVRGKKVTDLVAGELDHFYEGQKLRKQMKENRRIVAEQLLGVSKVMEDFAKEIQRE
RENHQVQEEQILQAFRDFGVEIEHVDIYCLDRGSIDIEMLIPVASNEHGECEKLVAPMLS
DILKENIVVKHEEKSSYPNGHSLISFGSAKTYSLDTGLATAAKGGGFVSGDSYAMMDLS
VGKYALAISDGMGNGQRAHMESKETVKLLQKILQSGIDEEIAIKSINSILSLRTTEEMFTT
LDLAMVDLRDASAKFLKIGSTPSFVKRANNILKIEASNLPMGIIEDVEVDVVGEQLKTGD
ILIMMSDGIFEGAQHVENHELWMKRKIKELQTEDPQEIADIIMEEVIRSGDGYINDDMTIV
VAKVKKNMPKWATIPIVGMQAQ

[0302] Coding sequence of *spoIIE* from *Bacillus anthracis* (SEQ ID NO:17):

```
   1 atgaagtttg agcaagtttt ctttagatgg ggatttatta ttgttgttat tggttttctt
  61 ttaggacgag catatatatt aacaaacatt ttaccgtttg cactgccgtt ttttgctgct
 121 gtttatgtta tgaagcggga taaaatgccg cttgcattct tggccctaat ggggggcgca
 181 ctgtcagttt cgatagataa tttattcttt acctttgcat ctatttttac tttcttcatt
 241 tataatatct tctttagtcg gtttacacgt aaaactgttg gacttgttcc attccaagta
 301 tttatctccg cattaaccgc acatttagtt gtcgtatatt ttgcgcaaca aacagtcacc
 361 atgtacgatt tacttgttag tacaattgag gccgggctta gcttcgtatt aactatgata
 421 tttttacaaa gtgtcccgct tttagtagaa agaaaaggga aacaacaagc cttagagaca
 481 gaagaaattg tttgtttaat tatattacta gcatctgttt taacaggtac aacagattgg
 541 ttcgtatatg acgcttctat tcaacatatt tttactaggt atttagtgct tgtatttgcg
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601 tttatcgcag gagcggctac aggatctaca gtaggggtcg tcactgggtt aatattaagc
661 ctggcaaatg tctccagttt gtcccaactt agcctacttg ctttttctgg attgcttggt
721 ggtttgttaa aagaagggaa gcgcataggt gttagtttag gtttattaat tgggacaagc
781 ctgattacgc tatatgtaga caagcaaaca aacattgtga caactttaat cgaatctggt
841 gtggcgattg ctttcttctt attaacaccg aaacttgtta tggatcgtat tgctaaattt
901 atgccaggca cacaggagca ttcgcaagat caacaacagt atttaagaag gatgcgtgat
961 gttacagcga ataaaattaa tcaatttgct aatgtgtttg ctgctttatc taatagcttt
1021 tctgtatatg gatacgtgga ggaagaagat aaagagacgg aggcagatct gttcttaagt
1081 acaattactg caaaaacatg tcaaacatgc tttaaaaagg accaatgctg ggtagttaat
1141 ttcgataaaa catacgatta tatgaaacaa ataatgagtg aaacagaaga ggggacgtta
1201 cagcataatc ggaagttagt tcgtgaatgg gataagcatt gtgtgagagg aaagaaagtg
1261 acggatttag tggcaggcga attagatcac ttctatgagg gacaaaaatt aagaaaacaa
1321 atgaaagaaa atcgtagaat agtagcggag caactattgg gtgtatcaaa agttatggag
1381 gattttgcta aggagataca aagagagcga gaaaaccatc aagtacagga agaacagatt
1441 ctgcaagcgt ttcgtgattt tggtgtagaa atagagcatg ttgatattta ttgtttagat
1501 agaggaagta ttgatattga aatgttgatt ccagttgcat ctaatgaaca cggagagtgt
1561 gaaaaattag ttgcaccgat gctttctgat attctaaagg aaaatatcgt tgttaagcat
1621 gaagaaaaat cttcttatcc gaatggccac agcttaatat catttggttc agcaaaaacg
1681 tattctcttg atacgggctt agccacagct gcaaaaggcg gtgggtttgt ttcaggtgat
1741 tcttatgcga tgatggattt aagtgttggt aaatatgctc ttgcgattag tgatggtatg
1801 ggaaatgggc aaagagctca tatggagagt aaagaaacag tgaaattatt acaaaaaata
1861 cttcaatcag gcattgatga agaaatagcg attaagtcta ttaactctat tctttcttta
1921 agaacaacag aagagatgtt tactacgtta gatttagcta tggtagattt gcgggatgcg
1981 agtgcgaagt ttttaaagat tggatcgacg ccgagttttg ttaaacgcgc aaacaatatt
2041 ttgaaaattg aagcaagtaa tttgccgatg ggaataattg aggatgttga agttgatgta
2101 gtgggtgagc aattaaaaac aggcgatatc cttattatga tgagcgatgg gatttttgag
2161 ggagcgcaac atgtggagaa tcatgaatta tggatgaagc gtaaaattaa agagttgcaa
2221 actgaagatc cgcaagaaat cgctgatatc atcatggaag aggtaattcg ctctggtgat
2281 ggttatataa atgatgatat gactattgta gtggcaaaag tgaagaaaaa tatgccgaag
2341 tgggctacca ttccaattgt gggaatgcag gcacaataa

[0303] Protein sequence of Protective Antigen (PagA) from *Bacillus anthracis* (SEQ ID NO:18):

MKKRKVLIPLMALSTILVSSTGNLEVIQAEVKQENRLLNESESSSQGLLGYYFSDLNFQA
PMVVTSSTTGDLSIPSSELENIPSENQYFQSAIWSGFIKVKKSDEYTFATSADNHVTMWV

DDQEVINKASNSNKIRLEKGRLYQIKIQYQRENPTEKGLDFKLYWTDSQNKKEVISSDNL
QLPELKQKSSNSRKKRSTSAGPTVPDRDNDGIPDSLEVEGYTVDVKNKRTFLSPWISNIH
EKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNVSPEARHPLVAAYPIVHVDMENIIL
SKNEDQSTQNTDSQTRTISKNTSTSRTHTSEVHGNAEVHASFFDIGGSVSAGFSNSNSST
VAIDHSLSLAGERTWAETMGLNTADTARLNANIRYVNTGTAPIYNVLPTTSLVLGKNQT
LATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDDFSSTPITMNYNQFLELEKTKQLRL
DTDQVYGNIATYNFENGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERRIAAVNP
SDPLETTKPDMTLKEALKIAFGFNEPNGNLQYQGKDITEFDFNFDQQTSQNIKNQLAELN
ATNIYTVLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLLN
IDKDIRKILSGYIVEIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNP
NYKVNVYAVTKENTIINPSENGDTSTNGIKKILIFSKKGYEIG

[0304] Nucleotide sequence encoding Protective Antigen (PagA) from *Bacillus anthracis* (SEQ ID NO:19):

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   1 atgaaaaaac gaaaagtgtt aataccatta atggcattgt ctacgatatt agtttcaagc
  61 acaggtaatt tagaggtgat tcaggcagaa gttaaacagg agaaccggtt attaaatgaa
 121 tcagaatcaa gttcccaggg gttactagga tactatttta gtgatttgaa ttttcaagca
 181 cccatggtgg ttacctcttc tactacaggg gatttatcta ttcctagttc tgagttagaa
 241 aatattccat cggaaaacca atattttcaa tctgctattt ggtcaggatt tatcaaagtt
 301 aagaagagtg atgaatatac atttgctact tccgctgata atcatgtaac aatgtgggta
 361 gatgaccaag aagtgattaa taaagcttct aattctaaca aaatcagatt agaaaaagga
 421 agattatatc aaataaaaat tcaatatcaa cgagaaaatc ctactgaaaa aggattggat
 481 ttcaagttgt actggaccga ttctcaaaat aaaaaagaag tgatttctag tgataactta
 541 caattgccag aattaaaaca aaaatcttcg aactcaagaa aaaagcgaag tacaagtgct
 601 ggacctacgg ttccagaccg tgacaatgat ggaatccctg attcattaga ggtagaagga
 661 tatacggttg atgtcaaaaa taaaagaact tttctttcac catggatttc taatattcat
 721 gaaaagaaag gattaaccaa atataaatca tctcctgaaa aatggagcac ggcttctgat
 781 ccgtacagtg atttcgaaaa ggttacagga cggattgata agaatgtatc accagaggca
 841 agacaccccc ttgtggcagc ttatccgatt gtacatgtag atatggagaa tattattctc
 901 tcaaaaaatg aggatcaatc cacacagaat actgatagtc aaacgagaac aataagtaaa
 961 aatacttcta caagtaggac acatactagt gaagtacatg gaaatgcaga agtgcatgcg
1021 tcgttctttg atattggtgg gagtgtatct gcaggattta gtaattcgaa ttcaagtacg
1081 gtcgcaattg atcattcact atctctagca ggggaaagaa cttgggctga aacaatgggt
1141 ttaaataccg ctgatacagc aagattaaat gccaatatta gatatgtaaa tactgggacg
```

1201 gctccaatct acaacgtgtt accaacgact tcgttagtgt taggaaaaaa tcaaacactc
1261 gcgacaatta aagctaagga aaaccaatta agtcaaatac ttgcacctaa taattattat
1321 ccttctaaaa acttggcgcc aatcgcatta aatgcacaag acgatttcag ttctactcca
1381 attacaatga attacaatca atttcttgag ttagaaaaaa cgaaacaatt aagattagat
1441 acggatcaag tatatgggaa tatagcaaca tacaattttg aaaatggaag agtgagggtg
1501 gatacaggct cgaactggag tgaagtgtta ccgcaaattc aagaaacaac tgcacgtatc
1561 atttttaatg gaaaagattt aaatctggta gaaaggcgga tagcggcggt taatcctagt
1621 gatccattag aaacgactaa accggatatg acattaaaag aagcccttaa aatagcattt
1681 ggatttaacg aaccgaatgg aaacttacaa tatcaaggga aagacataac cgaatttgat
1741 tttaatttcg atcaacaaac atctcaaaat atcaagaatc agttagcgga attaaacgca
1801 actaacatat atactgtatt agataaaatc aaattaaatg caaaaatgaa tattttaata
1861 agagataaac gttttcatta tgatagaaat aacatagcag ttggggcgga tgagtcagta
1921 gttaaggagg ctcatagaga agtaattaat tcgtcaacag agggattatt gttaaatatt
1981 gataaggata taagaaaaat attatcaggt tatattgtag aaattgaaga tactgaaggg
2041 cttaaagaag ttataaatga cagatatgat atgttgaata tttctagttt acggcaagat
2101 ggaaaaacat ttatagattt taaaaaatat aatgataaat taccgttata tataagtaat
2161 cccaattata aggtaaatgt atatgctgtt actaaagaaa acactattat taatcctagt
2221 gagaatgggg atactagtac caacgggatc aagaaaattt taatcttttc taaaaaaggc
2281 tatgagatag gataa

[0305] Protein sequence of Lethal Factor (Lef) from *Bacillus anthracis* (SEQ ID NO:20):

MNIKKEFIKVISMSCLVTAITLSGPVFIPLVQGAGGHGDVGMHVKEKEKNKDENKRKDE
ERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSDVLEMYKAIGGKIYIVD
GDITKHISLEALSEDKKKIKDIYGKDALLHEHYVVYAKEGYEPVLVIQSSEDYVENTEKAL
NVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFL
EQNSNEVQEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLSLEELKDQRM
LARYEKWEKIKQHYQHWSDSLSEEGRGLLKKLQIPIEPKKDDIIHSLSQEEKELLKRIQID
SSDFLSTEEKEFLKKLQIDIRDSLSEEEKELLNRIQVDSSNPLSEKEKEFLKKLKLDIQPYDI
NQRLQDTGGLIDSPSINLDVRKQYKRDIQNIDALLHQSIGSTLYNKIYLYENMNINNLTA
TLGADLVDSTDNTKINRGIFNEFKKNFKYSISSNYMIVDINERPALDNERLKWRIQLSPDT
RAGYLENGKLILQRNIGLEIKDVQIIKQSEKEYIRIDAKVVPKSKIDTKIQEAQLNINQEW
NKALGLPKYTKLITFNVHNRYASNIVESAYLILNEWKNNIQSDLIKKVTNYLVDGNGRF
VFTDITLPNIAEQYTHQDEIYEQVHSKGLYVPESRSILLHGPSKGVELRNDSEGFIHEFGH

AVDDYAGYLLDKNQSDLVTNSKKFIDIFKEEGSNLTSYGRTNEAEFFAEAFRLMHSTDH AERLKVQKNAPKTFQFINDQIKFIINS

[0306] Nucleotide sequence encoding Lethal Factor (Lef) from *Bacillus anthracis* (SEQ ID NO:21):

```
   1 atgaatataa aaaaagaatt tataaaagta attagtatgt catgtttagt aacagcaatt
  61 actttgagtg gtcccgtctt tatccccctt gtacaggggg cgggcggtca tggtgatgta
 121 ggtatgcacg taaaagagaa agagaaaaat aaagatgaga ataagagaaa agatgaagaa
 181 cgaaataaaa cacaggaaga gcatttaaag gaaatcatga aacacattgt aaaaatagaa
 241 gtaaaagggg aggaagctgt taaaaaagag gcagcagaaa agctacttga gaaagtacca
 301 tctgatgttt tagagatgta taaagcaatt ggaggaaaga tatatattgt ggatggtgat
 361 attacaaaac atatatcttt agaagcatta tctgaagata agaaaaaaat aaaagacatt
 421 tatgggaaag atgctttatt acatgaacat tatgtatatg caaaagaagg atatgaaccc
 481 gtacttgtaa tccaatcttc ggaagattat gtagaaaata ctgaaaaggc actgaacgtt
 541 tattatgaaa taggtaagat attatcaagg gatattttaa gtaaaattaa tcaaccatat
 601 cagaaatttt tagatgtatt aaataccatt aaaaatgcat ctgattcaga tggacaagat
 661 cttttattta ctaatcagct taaggaacat cccacagact tttctgtaga attcttggaa
 721 caaaatagca atgaggtaca agaagtattt gcgaaagctt ttgcatatta tatcgagcca
 781 cagcatcgtg atgttttaca gctttatgca ccggaagctt ttaattacat ggataaattt
 841 aacgaacaag aaataaatct atccttggaa gaacttaaag atcaacggat gctggcaaga
 901 tatgaaaaat gggaaaagat aaaacagcac tatcaacact ggagcgattc tttatctgaa
 961 gaaggaagag gacttttaaa aaagctgcag attcctattg agccaaagaa agatgacata
1021 attcattctt tatctcaaga agaaaaagag cttctaaaaa gaatacaaat tgatagtagt
1081 gatttttttat ctactgagga aaaagagttt ttaaaaaagc tacaaattga tattcgtgat
1141 tctttatctg aagaagaaaa agagctttta aatagaatac aggtggatag tagtaatcct
1201 ttatctgaaa aagaaaaaga gtttttaaaa aagctgaaac ttgatattca accatatgat
1261 attaatcaaa ggttgcaaga tacaggaggg ttaattgata gtccgtcaat taatcttgat
1321 gtaagaaagc agtataaaag ggatattcaa aatattgatg ctttattaca tcaatccatt
1381 ggaagtacct tgtacaataa aatttatttg tatgaaaata tgaatatcaa taaccttaca
1441 gcaaccctag gtgcggattt agttgattcc actgataata ctaaaattaa tagaggtatt
1501 ttcaatgaat tcaaaaaaaa tttcaaatat agtatttcta gtaactatat gattgttgat
1561 ataaatgaaa ggcctgcatt agataatgag cgtttgaaat ggagaatcca attatcacca
1621 gatactcgag caggatattt agaaaatgga aagcttatat tacaaagaaa catcggtctg
1681 gaaataaagg atgtacaaat aattaagcaa tccgaaaaag aatatataag gattgatgcg
1741 aaagtagtgc caaagagtaa aatagataca aaaattcaag aagcacagtt aaatataaat
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1801 caggaatgga ataaagcatt agggttacca aaatatacaa agcttattac attcaacgtg
1861 cataatagat atgcatccaa tattgtagaa agtgcttatt taatattgaa tgaatggaaa
1921 aataatattc aaagtgatct tataaaaaag gtaacaaatt acttagttga tggtaatgga
1981 agatttgttt ttaccgatat tactctccct aatatagctg aacaatatac acatcaagat
2041 gagatatatg agcaagttca ttcaaaaggg ttatatgttc cagaatcccg ttctatatta
2101 ctccatggac cttcaaaagg tgtagaatta aggaatgata gtgagggttt tatacacgaa
2161 tttggacatg ctgtggatga ttatgctgga tatctattag ataagaacca atctgattta
2221 gttacaaatt ctaaaaaatt cattgatatt tttaaggaag aagggagtaa tttaacttcg
2281 tatgggagaa caaatgaagc ggaatttttt gcagaagcct ttaggttaat gcattctacg
2341 gaccatgctg aacgtttaaa agttcaaaaa aatgctccga aaactttcca atttattaac
2401 gatcagatta agttcattat taactcataa
```

[0307] Protein sequence of Edema Factor (Cya) from *Bacillus anthracis* (SEQ ID NO:22):

MTRNKFIPNKFSIISFSVLLFAISSSQAIEVNAMNEHYTESDIKRNHKTEKNKTEKEKFKD
SINNLVKTEFTNETLDKIQQTQDLLKKIPKDVLEIYSELGGEIYFTDIDLVEHKELQDLSEE
EKNSMNSRGEKVPFASRFVFEKKRETPKLIINIKDYAINSEQSKEVYYEIGKGISLDIISKD
KSLDPEFLNLIKSLSDDSDSSDLLFSQKFKEKLELNNKSIDINFIKENLTEFQHAFSLAFSY
YFAPDHRTVLELYAPDMFEYMNKLEKGGFEKISESLKKEGVEKDRIDVLKGEKALKAS
GLVPEHADAFKKIARELNTYILFRPVNKLATNLIKSGVATKGLNVHGKSSDWGPVAGYI
PFDQDLSKKHGQQLAVEKGNLENKKSITEHEGEIGKIPLKLDHLRIEELKENGIILKGKKE
IDNGKKYYLLESNNQVYEFRISDENNEVQYKTKEGKITVLGEKFNWRNIEVMAKNVEG
VLKPLTADYDLFALAPSLTEIKKQIPQKEWDKVVNTPNSLEKQKGVTNLLIKYGIERKPD
STKGTLSNWQKQMLDRLNEAVKYTGYTGGDVVNHGTEQDNEEFPEKDNEIFIINPEGEF
ILTKNWEMTGRFIEKNITGKDYLYYFNRSYNKIAPGNKAYIEWTDPITKAKINTIPTSAEFI
KNLSSIRRSSNVGVYKDSGDKDEFAKKESVKKIAGYLSDYYNSANHIFSQEKKRKISIFR
GIQAYNEIENVLKSKQIAPEYKNYFQYLKERITNQVQLLLTHQKSNIEFKLLYKQLNFTE
NETDNFEVFQKIIDEK

[0308] Nucleotide sequence encoding Edema Factor (Cya) from *Bacillus anthracis* (SEQ ID NO:23):

```
   1 atgactagaa ataaatttat acctaataag tttagtatta tatcctttc agtattacta
  61 tttgctatat cctcctcaca ggctatagaa gtaaatgcta tgaatgaaca ttacactgag
 121 agtgatatta aaagaaacca taaaactgaa aaaaataaaa ctgaaaaaga aaaatttaaa
 181 gacagtatta ataacttagt taaaacagaa tttaccaatg aaactttaga taaaatacag
```

241 cagacacaag acttattaaa aaagatacct aaggatgtac ttgaaattta tagtgaatta
301 ggaggagaaa tctattttac agatatagat ttagtagaac ataaggagtt acaagattta
361 agtgaagaag agaaaaatag tatgaatagt agaggtgaaa aagttccgtt tgcatcccgt
421 tttgtatttg aaaagaaaag ggaaacacct aaattaatta taaatatcaa agattatgca
481 attaatagtg aacaaagtaa agaagtatat tatgaaattg gaaaggggat ttctcttgat
541 attataagta aggataaatc tctagatcca gagtttttaa atttaattaa gagtttaagc
601 gatgatagtg atagtagcga ccttttattt agtcaaaaat ttaaagagaa gctagaattg
661 aataataaaa gtatagatat aaattttata aaagaaaatt taactgaatt tcagcatgcg
721 ttttctttag cgttttctta ttattttgca cctgaccata gaacggtatt agagttatat
781 gcccccgaca tgtttgagta tatgaataag ttagaaaaag ggggatttga gaaaataagt
841 gaaagtttga agaaagaagg tgtggaaaaa gataggattg atgtgctgaa aggagaaaaa
901 gcacttaaag cttcaggttt agtaccagaa catgcagatg cttttaaaaa aattgctaga
961 gaattaaata catatattct ttttaggcct gttaataagt tagctacaaa ccttattaaa
1021 agtggtgtgg ctacaaaggg attgaatgtt catggaaaga gttcggattg gggccctgta
1081 gctggataca taccatttga tcaagattta tctaagaagc atggtcaaca attagctgtc
1141 gagaaaggaa atttagaaaa taaaaaatca attacagagc atgaaggtga aataggtaaa
1201 ataccattaa agttagacca tttaagaata gaagagttaa aggaaaatgg gataattttg
1261 aagggtaaaa aagaaattga taatggtaaa aaatattatt tgttagaatc gaataatcag
1321 gtatatgaat ttagaattag cgatgaaaac aacgaagtac aatacaagac aaaagaaggt
1381 aaaattactg ttttagggga aaaattcaat tggagaaata tagaagtgat ggctaaaaat
1441 gtagaagggg tcttgaagcc gttaacagct gactatgatt tatttgcact tgccccaagt
1501 ttaacagaaa taaaaaaaca aataccacaa aaagaatggg ataaagtagt taacacccca
1561 aattcattag aaaagcaaaa aggtgttact aatttattga ttaaatatgg aattgagagg
1621 aaaccggatt caactaaggg aactttatca aattggcaaa aacaaatgct tgatcgtttg
1681 aatgaagcag tcaaatatac aggatataca gggggggatg tggttaacca tggcacagag
1741 caagataatg aagagtttcc tgaaaaagat aacgaaattt ttataattaa tccagaaggt
1801 gaatttatat taactaaaaa ttgggagatg acaggtagat ttatagaaaa aaacattacg
1861 ggaaaagatt atttatatta ttttaaccgt tcttataata aaatagctcc tggtaataaa
1921 gcttatattg agtggactga tccgattaca aaagccaaaa taaataccat ccctacgtca
1981 gcagagttta taaaaaactt atccagtatc agaagatctt caaatgtagg agtttataaa
2041 gatagtggcg acaaagacga atttgcaaaa aaagaaagcg tgaaaaaaat tgcaggatat
2101 ttgtcagact attacaattc agcaaatcat atttttctc aggaaaaaaa gcgtaaaata
2161 tcaatatttc gtggaatcca agcctataat gaaattgaaa atgttctaaa atctaaacaa
2221 atagcaccag aatacaaaaa ttattttcaa tatttaaagg aaaggattac caatcaagtt
2281 caattgcttc taacacatca aaaatctaat attgaattta aattattgta taaacaatta

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2341 aactttacag aaaatgaaac ggataatttt gaggtcttcc aaaaaattat tgatgaaaaa
2401 taa
```

[0309] Protein sequence of Lex Repressor (LexA) from *Bacillus anthracis* (SEQ ID NO:24):

MEKLTKRQQDILDFIKLKVQEKGYPPSVREIGQAVGLASSSTVHGHLSRLEEKGYIRRDP
TKPRAIEILGEDRMDTETQSVIQVPIVGKVTAGLPITAVESVEEHFPLPASIVAGADQVFM
LRISGDSMIEAGIFDGDLVVVRQQQSAYNGEIVVALTEDNEATVKRFYKEKDHFRLQPE
NSSLEPIILKQVSVIGKVIGVYRDLH

[0310] Nucleotide sequence encoding Lex Repressor (LexA) from *Bacillus anthracis* (SEQ ID NO:25):

```
   1 atggaaaagt taacgaaacg ccagcaagac attctcgact ttattaagct aaaagtacaa
  61 gaaaaaggat atccaccctc cgtacgtgaa atcggtcaag cagtcggcct cgcttctagt
 121 tctacagtgc acggacattt atcaagatta gaagaaaaag gatacattcg acgcgatcca
 181 acaaaaccac gtgcaattga aattttaggt gaagaccgaa tggatacaga aacacaatct
 241 gttattcaag ttccaattgt cggaaaagtt actgccggtt taccaattac agcggtcgaa
 301 agcgtagagg agcacttccc tcttccagct agcattgtcg caggagcaga tcaagtgttt
 361 atgttacgta tttccgggga tagtatgatt gaggctggca ttttcgatgg agatttagtt
 421 gttgttcgcc aacaacagtc tgcatataat ggtgaaattg tagtcgcttt aacagaagat
 481 aatgaagcaa ctgttaaacg tttctataaa gaaaaagacc atttccgtct acaaccggaa
 541 aactcttcat tagaacctat cattttaaag caagtgtcag ttatcggtaa agtaattggc
 601 gtatatcgtg atttacatta a
```

[0311] Protein sequence of RecA from *Bacillus anthracis* (SEQ ID NO:26):

MSDRQAALDMALKQIEKQFGKGSIMKLGEQAERKVSTVSSGSLALDVALGVGGYPRGR
IIEIYGPESSGKTTVSLHAIAEVQRQGGQAAFIDAEHAMDPVYAQKLGVNIDELLLSQPD
TGEQGLEIAEALVRSGAVDIIVIDSVAALVPKAEIEGDMGDSHVGLQARLMSQALRKLS
GAINKSKTIAIFINQIREKVGVMFGNPETTPGGRALKFYSTVRLEVRRAEQLKQGNDIVG
NKTKVKVVKNKVAPPFRVAEVDIMYGEGISREGEILDMASELDIVQKSGAWYSYNEER
LGQGRENSKQFLKENTDLREEIAFFIREHHGISEDSGAEGMEDPNLLD

[0312] Sequence from the *recA* gene of *Bacillus anthracis* (GenBank Acc. No. gi12656080 gb AF229167.1) (SEQ ID NO:27):

```
   1 atgagtgatc gtcaagcagc gttagatatg gcgttaaaac aaatagagaa gcaattcggt
  61 aaaggttcaa ttatgaaatt aggagaacaa gcagagcgca aagtttctac ggtttctagt
 121 ggttctttag cacttgatgt ggcattaggg gtaggcggat acccacgcgg ccgtattatc
```

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 181 gaaatttacg gacctgaaag ttcaggtaaa acaacagttt cattacacgc aattgcagaa
 241 gtacagcgtc aaggtggaca agcagcgttc attgatgctg agcatgcaat ggatcctgta
 301 tatgcacaaa aactaggtgt taacatcgat gaattactat tatcacaacc tgatacaggg
 361 gagcaaggtt tagaaatcgc agaagcactt gtacgaagtg gtgcggttga tattatcgta
 421 attgactctg tagcagctct tgtaccgaaa gctgaaattg aaggagacat gggtgactca
 481 cacgtaggtt tacaagctcg tctaatgtct caagcacttc gtaaactttc aggtgcaatc
 541 aataaatcaa aaacaatcgc aatctttatt aaccaaattc gtgaaaaagt tggggttatg
 601 ttcggaaatc aactacaagt tgcttaatcg taaagattaa ggagtttacc atgggtttcc
 661 cttgctagtg atagcatgtt aaaaaacctt gtgaacctta ttgcctaagg gtgtaactat
 721 tcatatagtt gctaacggtg aagcccacca ttattcaggg taataccgtg ccaagctcca
 781 tagtgatatg gggaaggtgt agagactacc gaaaaggact tttagttaac tgagtagggt
 841 acggcagatg ataggctact gctggaagtg caaggctctc tgtaaagaga tgaagagata
 901 gtccggacta tagagatgga aaatctatag atagtgccag aaacaactcc aggtggtcgt
 961 gcgttgaaat tctattcaac tgttcgtctt gaagtgcgtc gtgcggagca attaaaacaa
1021 ggtaacgaca tc
```

[0313] Coding sequence of the *uvrC* gene of *Bacillus anthracis* (SEQ ID NO:28):

```
ATGAAAGATAGGCAAGGAACGGTTATATATGTCGGAAAGGCAAAAGTGCTTAAAAA
TCGTGTGCGCTCGTACTTTACTGGTTCGCATGACGGGAAAACACTTCGGTTAGTAGG
AGAAATTGTAGATTTTGAATATATTGTAACCTCCTCAAATCTAGAGGCGCTCATTTT
GGAGTTAAACTTAATAAAAAAACATGACCCAAAATATAATATTCAATTAAAAGATG
ATAAAACATATCCTTTTATTAAAATTACAGCTGAGAAACAACCGCGCTTACTTATTA
CGCGAAATGTAAAAAAGGATAAAGGAAAGTATTTTGGCCCTTATCCGAATGCACAA
TCAGCTCATGAAACGAAAAAACTGCTGGATCGTATGTATCCGCTTCGTAAGTGCTCA
AATATGCCGGATAAAGTTTGTTTATATTATCATATGGGTCAATGTTTAGCACCTTGTG
TGAAAGAAGTGACGGAAGAACAAAATAAAGAAATTGTAGATGAGATTATTAAGTTT
TTAAATGGTGGGCATAAAGAAGTTCGTTCAGAATTAGAAACAAAAATGTATGAAGC
TTCAGAGAAACTAGAGTTTGAACGTGCAAAAGAGTTACGTGATCAAATCGCTCATAT
CGATGCGATTATGGAAAAACAAAAGATGATTATGAGTGATTTAGTGGACCGTGATG
TGTTTGGCTATGCAGTTGATAAAGGGTGGATGTGTGTTCAAGTTTTCTTCGTTCGGAA
AGGAAAGTTAATTGAACGTGATGTTTCTATGTTTCCAATATATGATGAACCAGAAGA
GGGATTCTTAACGTTTATCGGTCAATTTTATGAAAACAGCAGTCATTTTAAGCCGAA
AGAAATAGTTGTTCCAGGAAGTATAGACTCAGAATTAGTAGAACGCTTTTTAGAAGT
GGAAGCGACACAGCCGAAACGCGGTAAGAAAAAAGATCTTGTAGAACTGGCAAAT
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AAAAATGCGAAGATTGCCCTGGAAGAGAAATTCTATTTAATTGAACGTGATGAAGA
GCGAACGATTAAAGCTGTAGAGAATTTAGGGAAGCAGCTCGGAATTGAAACGCCTT
ATCGTATTGAAGCATTTGATAACTCAAATATTCAAGGGACAAATCCTGTTTCTGCAA
TGATTGCTTTTATTGATGGGAAACCAGCTAAGAAAGAATACAGGAAATATAAAATT
AAAACAGTTCAAGGACCAGATGATTATGAGTCTATGAGAGAAGTTGTGAGACGCCG
TTATACAAGGGCGCTGAAAGAGGGTTTACCTTTACCAGATTTAATCATTATTGATGG
CGGAAAAGGTCATCTGGCGGCTGCAAGTGATGTTCTAGAAAATGAGCTCGGTTTATA
TATTCCGATGGCAGGTCTTGTAAAAGATGACAAACATAAAACATCTCATTTAATTAT
TGGAGATCCACCTGAACCTGTGATGCTGGAGAGAAATAGCCAAGAATTTTATTTATT
GCAGCGTGTTCAAGATGAAGTGCATCGATTTGCAATTACATTTCATCGTCAATTACA
CGGGAAATCTGTCATTCAATCAGCACTGGATGATATTCCTGGAATCGGTGATAAACG
GAAAAAGGTATTGTTAAAACATTTTGGTTCATTAAAGAAGATGAAAGAAGCTTCTAT
AGAGGAATTTGTCGAAGCAGGTATGCCGAAAAATGTCGCAGAGACGATTTATACTT
ATTTAACAGATAAGAAGACGTTG

[0314] The following nucleotide sequence is that of the wild type *E. coli* RecA coding sequence (from GenBank Acc. No. V00328) (SEQ ID NO:50):

```
   1 atggctatcg acgaaaacaa acagaaagcg ttggcggcag cactgggcca gattgagaaa
  61 caatttggta aaggctccat catgcgcctg ggtgaagacc gttccatgga tgtggaaacc
 121 atctctaccg gttcgctttc actggatatc gcgcttgggg caggtggtct gccgatgggc
 181 cgtatcgtcg aaatctacgg accggaatct tccggtaaaa ccacgctgac gctgcaggtg
 241 atcgccgcag cgcagcgtga aggtaaaacc tgtgcgttta tcgatgctga acacgcgctg
 301 gacccaatct acgcacgtaa actgggcgtc gatatcgaca acctgctgtg ctcccagccg
 361 gacaccggcg agcaggcact ggaaatctgt gacgccctgg cgcgttctgg cgcagtagac
 421 gttatcgtcg ttgactccgt ggcggcactg acgccgaaag cggaaatcga aggcgaaatc
 481 ggcgactctc acatgggcct tgcggcacgt atgatgagcc aggcgatgcg taagctggcg
 541 ggtaacctga agcagtccaa cacgctgctg atcttcatca accagatccg tatgaaaatt
 601 ggtgtgatgt tcggtaaccc ggaaaccact accggtggta acgcgctgaa attctacgcc
 661 tctgttcgtc tcgacatccg tcgtatcggc gcggtgaaag agggcgaaaa cgtggtgggt
 721 agcgaaaccc gcgtgaaagt ggtgaagaac aaaatcgctg cgccgtttaa acaggctgaa
 781 ttccagatcc tctacggcga aggtatcaac ttctacggcg aactggttga cctgggcgta
 841 aaagagaagc tgatcgagaa agcaggcgcg tggtacagct acaaaggtga gaagatcggt
 901 cagggtaaag cgaatgcgac tgcctggctg aaagataacc cggaaaccgc gaaagagatc
```

```
 961 gagaagaaag tacgtgagtt gctgctgagc aacccgaact caacgccgga tttctctgta
1021 gatgatagcg aaggcgtagc agaaactaac gaagattttt aa
```

[0315] The wild type *E. coli* RecA amino acid sequence is as follows (from GenBank Acc. No. V00328) (SEQ ID NO:51):

MAIDENKQKALAAALGQIEKQFGKGSIMRLGEDRSMDVETISTGSLSLDIALGAGGLPM
GRIVEIYGPESSGKTTLTLQVIAAAQREGKTCAFIDAEHALDPIYARKLGVDIDNLLCSQP
DTGEQALEICDALARSGAVDVIVVDSVAALTPKAEIEGEIGDSHMGLAARMMSQAMRK
LAGNLKQSNTLLIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIRRIGAVKEGENV
VGSETRVKVVKNKIAAPFKQAEFQILYGEGINFYGELVDLGVKEKLIEKAGAWYSYKGE
KIGQGKANATAWLKDNPETAKEIEKKVRELLLSNPNSTPDFSVDDSEGVAETNEDF

[0316] The amino acid sequence of a predicted temperature sensitive *E. coli* RecA protein is as follows (SEQ ID NO:52) (the mutation relative to the GenBank Acc. No. V00328 wild-type sequence is underlined in the sequence):

MAIDENKQKALAAALGQIEKQFGKGSIMRLGEDRSMDVETISTGSLSLDIALGAGGLPM
GRIVEIYGPESSGKTTLTLQVIAAAQREGKTCAFIDAEHALDPIYARKLGVDIDNLLCSQP
DTGEQALEICDALARSGAVDVIVVDSVAALTPKAEIEGEIGDSHMGLAARMMSQAMRK
LAGNLKQSNTLLIFINQIRMKIGVMFGNPETTTGGNALKFYASVRLDIRRIGAVKEGENV
VGSETRVKMVKNKIAAPFKQAEFQILYGEGINFYGELVDLGVKEKLIEKAGAWYSYKG
EKIGQGKANATAWLKDNPETAKEIEKKVRELLLSNPNSTPDFSVDDSEGVAETNEDF

[0317] The wild type *B. anthracis* RecA (GenBank Acc. No. NC_007530) coding region, which in the *B. anthracis* genome occurs in two segments, separated by an intron, has the following sequence (after the intron is removed in silico) (SEQ ID NO:53):

```
   1 atgagtgatc gtcaagcagc gttagatatg gcgttaaaac aaatagagaa gcaattcggt
  61 aaaggttcaa ttatgaaatt aggagaacaa gcagagcgca aagtttctac ggtttctagt
 121 ggttctttag cacttgatgt ggcattaggg gtaggcggat acccacgcgg ccgtattatc
 181 gaaatttacg gacctgaaag ttcaggtaaa acaacagttt cattacacgc aattgcagaa
 241 gtacagcgtc aaggtggaca agcagcgttc attgatgctg agcatgcaat ggatcctgta
 301 tatgcacaaa aactaggtgt taacatcgat gaattactat tatcacaacc tgatacaggg
 361 gagcaaggtt tagaaatcgc agaagcactt gtacgaagtg gtgcggttga tattatcgta
 421 attgactctg tagcagctct tgtaccgaaa gctgaaattg aaggagacat gggtgactca
 481 cacgtaggtt tacaagctcg tctaatgtct caagcacttc gtaaactttc aggtgcaatc
 541 aataaatcaa aaacaatcgc aatctttatt aaccaaattc gtgaaaaagt tggggttatg
```

```
 601 ttcggaaatc cagaaacaac tccaggtggt cgtgcgttga aattctattc aactgttcgt
 661 cttgaagtgc gtcgtgcgga gcaattaaaa caaggtaacg acatcgttgg taataaaaca
 721 aaagtaaaag tagttaaaaa taaagtggca ccaccattcc gtgttgcgga agttgatatt
 781 atgtacggag aaggtatttc aagagaaggt gaaatcttag atatggcctc tgaacttgat
 841 atcgttcaaa agagcggtgc ttggtactct tataatgaag aacgcttagg acaaggtcgt
 901 gaaaattcga agcagttctt aaaagaaaat acggatttaa gagaagaaat tgccttcttt
 961 attcgtgagc atcacggaat tagcgaagat tctggtgctg aaggtatgga agatccaaat
1021 cttcttgatt aa
```

[0318] The amino acid sequence of a predicted temperature sensitive *B. anthracis* RecA protein comprising a mutation (underlined) analogous to that of the *E. Coli* recA44 temperature sensitive mutant is as follows (SEQ ID NO:54):

MSDRQAALDMALKQIEKQFGKGSIMKLGEQAERKVSTVSSGSLALDVALGVGGYPRGR
IIEIYGPESSGKTTVSLHAIAEVQRQGGQAAFIDAEHAMDPVYAQKLGVNIDELLLSQPD
TGEQGLEIAEALVRSGAVDIIVIDSVAALVPKAEIEGDMGDSHVGLQARLMSQALRKLS
GAINKSKTIAIFINQIREKVGVMFGNPETTPGGRALKFYSTVRLEVRRAEQLKQGNDIVG
NKTKVKMVKNKVAPPFRVAEVDIMYGEGISREGEILDMASELDIVQKSGAWYSYNEER
LGQGRENSKQFLKENTDLREEIAFFIREHHGISEDSGAEGMEDPNLLD

[0319] The region of the *B. anthracis* genome that includes the *uvrA* gene and *uvrB* gene appears below (SEQ ID NO:55) (coding regions are underlined; sequence from GenBank Acc. No. NC_007530). The UvrA protein and UvrB protein are encoded by the complement of the shown underlined sequence. Complement of the UvrA coding sequence is found at: 4,884,383 to 4,887,259. Complement of the UvrB coding sequence is found at: 4,887,265 to 4,889,241. A region of only five base pairs separates the two coding regions. Also shown are upstream and downstream sequences.

```
4884001 tcatccatac ttaataaact cgcatctttt attagtagta catcatcaca gtgttgatag
4884061 aagatgtagc aatccttttt cacttcatta tgaaacgcgt tctccgcttc ttctccctct
4884121 aaacacttct ttctctcata actaaatatg tgccatagat acccacatgc atgcttatct
4884181 ccataaagga aaatatcttc tttttcctca tcacttacat gatttgcaaa atgatcctcc
4884241 catcgcttcc gaaaatatac accccacttt tgaaactctc ttaccttcat atttttcttt
4884301 cgcaacatat ctaaaaactc catcttctcc ccctatacaa taaaaaccga gcgtcacttc
4884361 cactcggttt tcattcatac acttattgtg ataactctac ttcttttatt ttctctttca
4884421 ttctcgcttt atcacgattt aaaatctctt ttaaatactt acctgtatac gagcgctctt
4884481 ctttcactac ttgctctggc gttccggaag caacgatttg tccacctttg tctccgcctt
```

4884541 ctggtccaag gtcaacgata taatccgctg ttttaattac atctaaatta tgttcaatga
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4884661 catgcgcatg taaaccagtc gttggctcgt ctaaaatgta tagtgtacgt cctgtagaac
4884721 gacggtgtaa ttcagaagct aatttcacac gctgtgcttc accaccagat aaagtcgtgg
4884781 ctggttgccc taatttcata taaccaagcc caacgtctac aagcgtttga agtttacgtt
4884841 taatttttgg gatattagcg aagaactcta ctccgtcttc aatcgtcatc cctaacactt
4884901 cagaaatgtt tttatcttta tatttcactt ctaacgtttc acggttgtaa cgtttaccgt
4884961 gacaaacttc acacggaacg tatacgtctg gtaagaagtg catctcaatt ttaataattc
4885021 catcaccacg gcacgcttca caacgtccac cttttacgtt aaagctgaaa cgccctttt
4885081 gatatccgcg cactttcgct tcattcgttt gcgcaaacac atcacgaata tcatcgaaca
4885141 cacctgtata ggttgctgga ttagaacgtg gtgtacgacc gattggcgat tgatcaatat
4885201 cgataacttt atctaaatgc tcaagacctt taatttcttt atgagtacct ggcttcgctt
4885261 tcgctttata taacttttgc gctaacgatt tatatagtac ttcattaatc atcgtacttt
4885321 tacctgatcc agatacaccc gttaccgcta caaacgtacc aagcgggaat gacatcttcg
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4885441 gtctttcaag tggaactgga ataaactctt taccgcttaa atacttacct gttagtgaat
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4885681 cgttatcgcg ctgatgcaaa ccgatagaag gctcatcaag aatgtaaagc accccagtaa
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CLAIMS

What we claim is:

1. An isolated, asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair, wherein the strain is defective with respect to at least one DNA repair enzyme selected from the group consisting of UvrA, UvrB, UvrC, and RecA.

2. A bacterium of the *Bacillus anthracis* strain of claim 1, wherein the nucleic acid of the bacterium has been modified so that the bacteria are attenuated for proliferation.

3. A bacterium of the *Bacillus anthracis* strain of claim 2, wherein the nucleic acid of the bacterium has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation.

4. An isolated, asporogenic *Bacillus anthracis* bacterium that is attenuated for nucleic acid repair, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation.

5. The *Bacillus anthracis* bacterium of claim 4, wherein the bacterium has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation.

6. The *Bacillus anthracis* bacterium of claim 4, wherein the bacterium is defective with respect to at least one DNA repair enzyme.

7. The *Bacillus anthracis* strain of claim 3 or bacterium of claim 5, wherein the nucleic acid targeting compound is a psoralen compound activated by UVA irradiation.

8. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 4, which is defective with respect to SpoIIE.

9. The *Bacillus anthracis* strain of claim 8, which comprises a mutation in the *spoIIE* gene.

10. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 6, which is defective with respect to UvrA, UvrB, or both UvrA and UvrB.

11. The *Bacillus anthracis* strain of claim 10, which comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene.

12. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 6, wherein the strain is defective with respect to SpoIIE, UvrA, and UvrB.

13. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 6, which is defective with respect to UvrC.

14. The *Bacillus anthracis* strain of claim 13, which comprises a mutation in the *uvrC* gene.

15. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 6, which is defective with respect to RecA.

16. The *Bacillus anthracis* strain of claim 15, which comprises a mutation in the *recA* gene.

17. The *Bacillus anthracis* strain of claim 15, which is a repressible *recA* mutant.

18. The *Bacillus anthracis* strain of claim 17, which comprises a *recA* gene under control of the lac repressor.

19. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 6, which comprises a temperature-sensitive *recA* gene.

20. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 4, which expresses protective antigen under the control of an SOS regulatory sequence.

21. The *Bacillus anthracis* strain of claim 1 or bacterium of claim 4, which comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain or bacterium.

22. An isolated *Bacillus anthracis* strain comprising a heterologous expression cassette comprising a nucleic acid encoding protective antigen, wherein the nucleic acid encoding protective antigen is operably linked to an inducible promoter.

23. The *Bacillus anthracis* strain of claim 22, wherein expression of the protective antigen is induced by treatment of the strain with a psoralen and UVA.

24. The *Bacillus anthracis* strain of claim 22, wherein the nucleic acid encoding protective antigen is operably linked to an SOS regulatory sequence.

25. The *Bacillus anthracis* strain of claim 22, which comprises a mutation that attenuates the ability of the strain to repair its nucleic acid.

26. The *Bacillus anthracis* strain of claim 25, which is defective with respect to at least one DNA repair enzyme.

27. The *Bacillus anthracis* strain of claim 26, which is defective with respect to RecA.

28. The *Bacillus anthracis* strain of claim 27, which comprises a mutation in its *recA* gene.

29. The *Bacillus anthracis* strain of claim 27, which is a repressible *recA* mutant.

30. The *Bacillus anthracis* strain of claim 29, which comprises a *recA* gene that is under control of the lac repressor.

31. The *Bacillus anthracis* strain of claim 27, which comprises a temperature sensitive *recA* gene.

32. An isolated *Bacillus anthracis* strain, which is a temperature sensitive *recA* mutant, wherein the strain comprises a *recA* gene which comprises a mutation analogous to the V246M mutation of the recA44 temperature sensitive *recA* mutant of *E. coli*.

33. A *Bacillus anthracis* strain, which is a temperature sensitive *recA* mutant, wherein the strain comprises a recA gene that comprises the recA44(ts) allele of *E. coli*.

34. An isolated *Bacillus anthracis* strain, which is a repressible *recA* mutant.

35. The *Bacillus anthracis* strain of claim 34, which comprises a *recA* gene that is under control of the lac repressor.

36. The *Bacillus anthracis* strain of claim 34, which comprises an expression cassette that expresses a RecA antisense RNA.

37. The *Bacillus anthracis* strain of claim 32, 33, or 34, which is defective with respect to at least one additional DNA repair enzyme.

38. The *Bacillus anthracis* strain of claim 26 or 37, which is defective with respect to UvrA, UvrB, or both UvrA and UvrB.

39. The *Bacillus anthracis* strain of claim 38, comprises a mutation in the *uvrA* gene, the *uvrB* gene, or both the *uvrA* and *uvrB* gene.

40. The *Bacillus anthracis* strain of claim 26 or 37 which is defective with respect to UvrC.

41. The *Bacillus anthracis* strain of claim 40, which comprises a mutation in the *uvrC* gene.

42. The *Bacillus anthracis* strain of claim 22, 32, 33, or 34, which comprises one or more mutations in the *lef* gene, the *cya* gene, or both genes that decreases the toxicity of the strain.

43. The *Bacillus anthracis* strain of claim 22, 32, 33, or 34, wherein the nucleic acid of the bacteria of the strain has been modified so that the bacteria are attenuated for proliferation.

44. The *Bacillus anthracis* strain of claim 43, wherein the nucleic acid of the bacteria of the strain has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation.

45. The *Bacillus anthracis* strain of claim 44, wherein the nucleic acid targeting compound is a psoralen compound activated by UVA irradiation.

46. The *Bacillus anthracis* strain of claim 22, 32, 33, or 34, which is asporogenic.

47. The *Bacillus anthracis* strain of claim 46, which is defective with respect to SpoIIE.

48. The *Bacillus anthracis* strain of claim 47, which comprises a mutation in the *spoIIE* gene.

49. An isolated, sporulation-deficient *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid so that the bacterium is attenuated for proliferation.

50. An isolated, asporogenic *Bacillus anthracis* bacterium, wherein the nucleic acid of the bacterium has been modified so that the bacterium is attenuated for proliferation.

51. The bacterium of claim 50, wherein the bacterium has been modified with a nucleic acid targeting compound that reacts directly with the nucleic acid, so that the bacterium is attenuated for proliferation.

52. An isolated, sporulation-deficient *Bacillus anthracis* strain that is attenuated for nucleotide excision repair (NER).

53. An isolated, asporogenic *Bacillus anthracis* strain that is attenuated for nucleotide excision repair (NER).

54. An isolated *Bacillus anthracis* strain which is defective with respect to SpoIIE.

55. The *Bacillus anthracis* strain of claim 54, which is sporulation-deficient.

56. The *Bacillus anthracis* strain of claim 54, which is asporogenic.

57. A *Bacillus anthracis* strain, which is an inducible *recA* mutant.

58. A *Bacillus anthracis* strain comprising a nucleic acid encoding an antigen, wherein the nucleic acid encoding the antigen is operably linked to a heterologous promoter.

59. The *Bacillus anthracis* strain of claim 58, wherein the promoter is an inducible promoter.

60. A vaccine composition comprising the bacterium of claim 4, 49, or 50 or a bacterium from the *Bacillus anthracis* strain of claim 1, 22, 32, 33, 34, 52, 53, 54, 57, or 58.

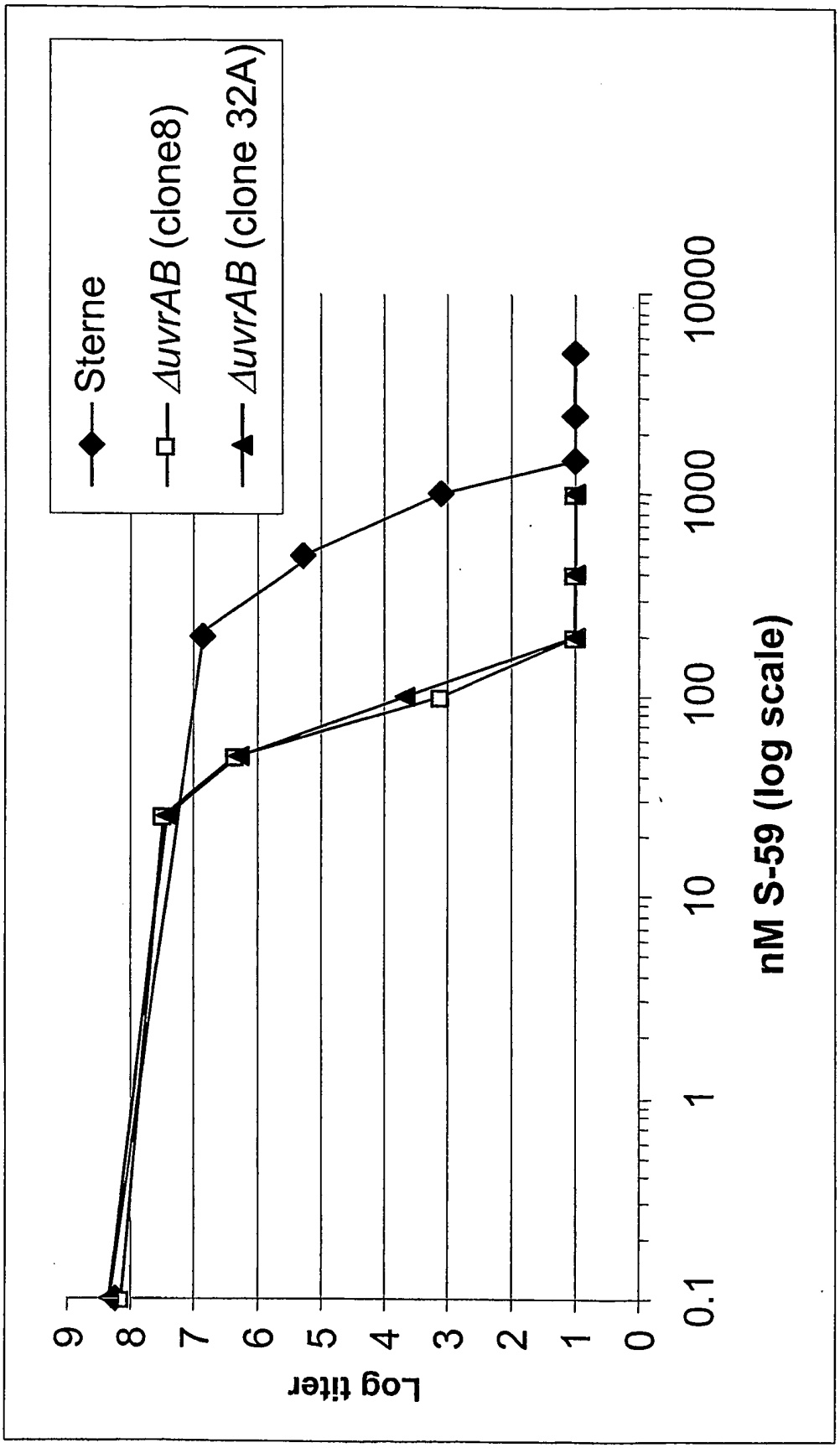
61. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising the bacterium of claim 4, 49, or 50 or a bacterium from the *Bacillus anthracis* strain of claim 1, 22, 32, 33, 34, 52, 53, 54, 57, or 58.

62. The use of the bacterium of claim 4, 49, or 50 or a bacterium from the *Bacillus anthracis* strain of claim 1, 22, 32, 33, 34, 52, 53, 54, 57, or 58 in the manufacture of a medicament for inducing an immune response in a host to *Bacillus anthracis*.

63. A vaccine composition comprising a bacterium from an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair.

64. A method of inducing an immune response in a host to *Bacillus anthracis* comprising administering to the host an effective amount of a composition comprising a bacterium from an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair.

65. The use of the bacterium from an asporogenic *Bacillus anthracis* strain that is attenuated for nucleic acid repair in the manufacture of a medicament for inducing an immune response in a host to *Bacillus anthracis*.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ΔuvrAB (clone 32A)
Log titer
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7
8
9
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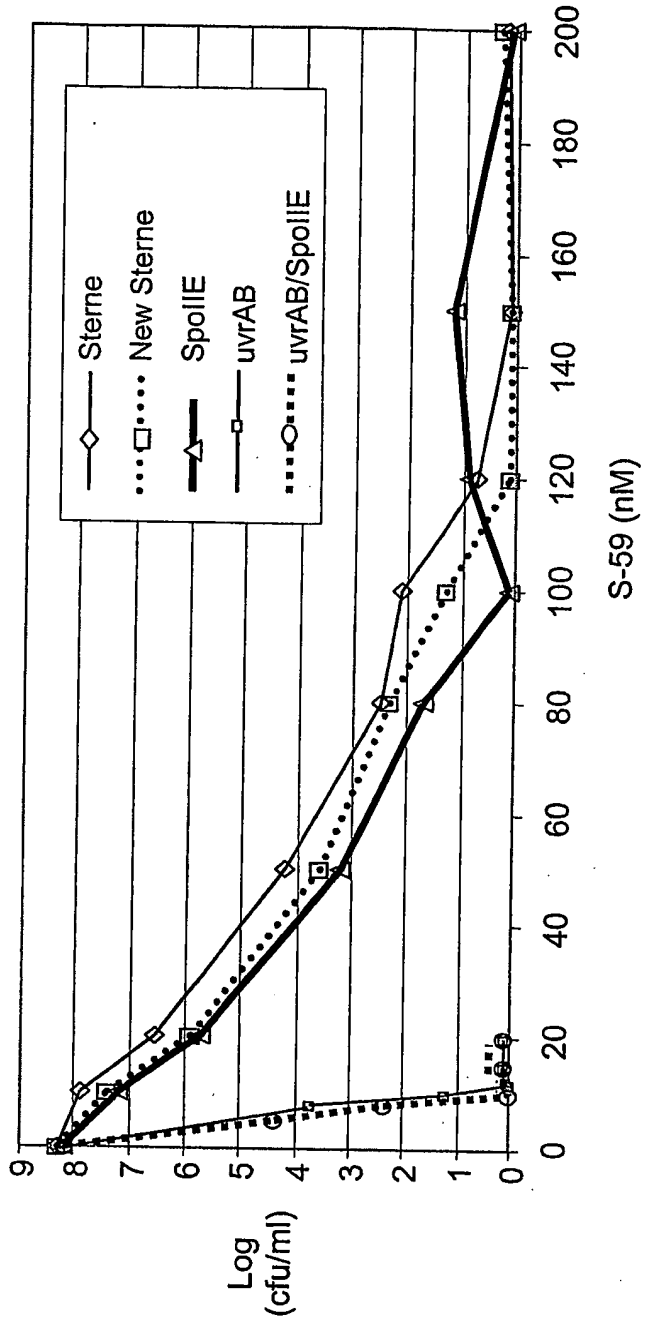
1
10
100
1000
10000
nM S-59 (log scale)

FIGURE 1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New Sterne
SpoIIE
uvrAB
uvrAB/SpoIIE
Log (cfu/ml)
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2
3
4
5
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7
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9
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20
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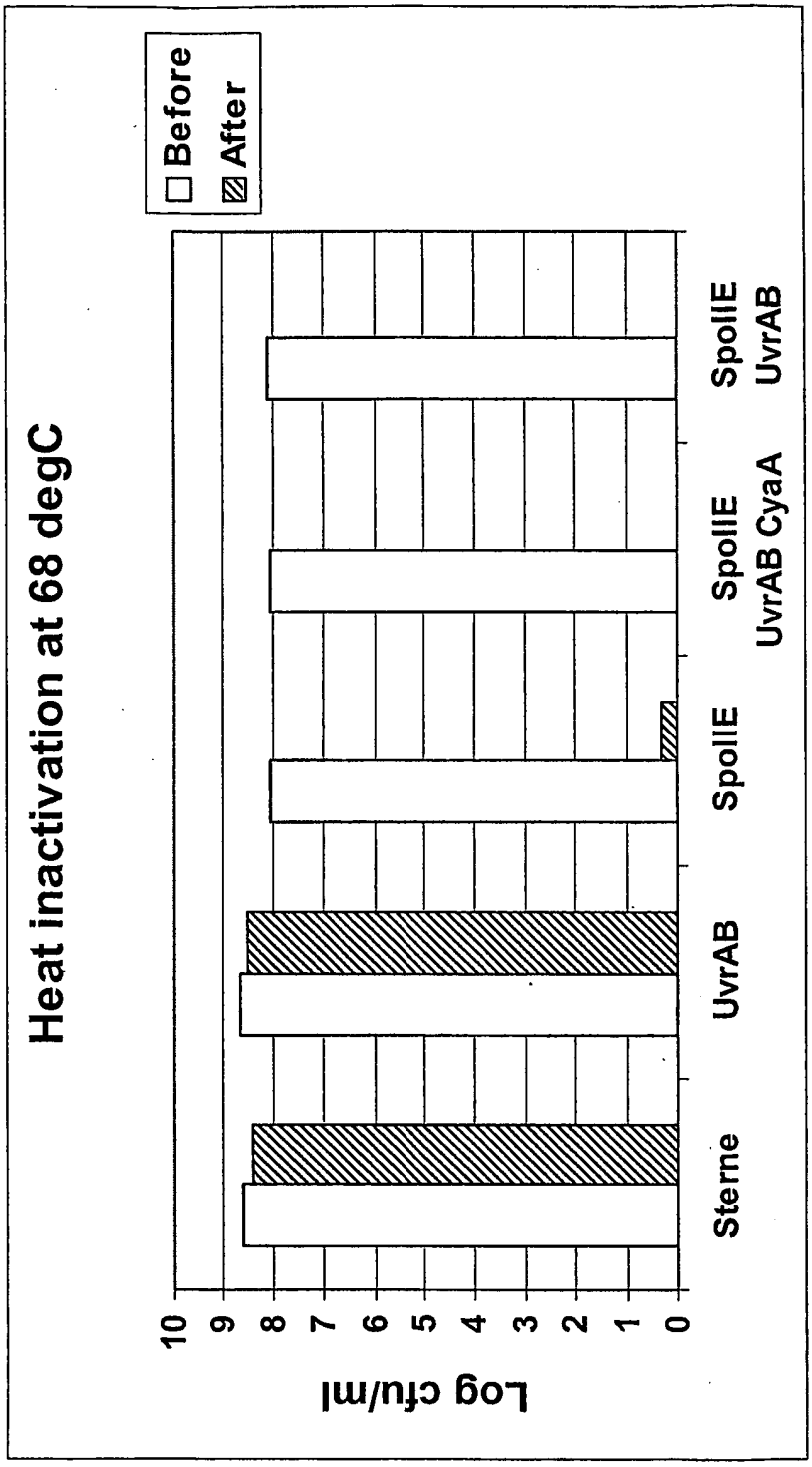
60
80
100
120
140
160
180
200
S-59 (nM)

FIGURE 2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After
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7
6
5
4
3
2
1
0
Sterne
UvrAB
SpoIIE
SpoIIE UvrAB CyaA
SpoIIE
UvrAB

FIGURE 3